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Role of TRAIL

(TNF-Related Apoptosis-Inducing Ligand)

in the onset and progression of type 1 diabetes mellitus

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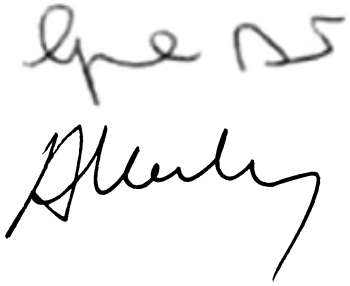

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A physician who fails to enter the body of a patient
with the lamp of knowledge and understanding
can never treat diseases.
He should first study all the factors,
including environment,
which influence a patient's disease,
and then prescribe treatment.
It is more important to prevent the occurrence of disease
than to seek a cure.

(Charaka Samhita, 1000 B.C.)

To all my young friends with diabetes

To uncle Mario

Role of TRAIL (TNF-Related Apoptosis-Inducing Ligand) in the onset and progression of type 1 diabetes mellitus

Ruolo di TRAIL (TNF-Related Apoptosis-Inducing Ligand) nell'esordio e nella progressione del diabete mellito di tipo 1

CONTENTS

PREFACE

In April 2011, I heard for the first time Professor Giorgio Zauli talking about TNF-related apoptosis-inducing ligand (TRAIL) during his lecture at the final examination of the PhD students in Reproductive Sciences and Development at the University of Trieste. While listening that the administration of recombinant TRAIL ameliorated the severity of streptozocin-induced type 1 diabetes in mice, I was captured by this potential new therapeutic chance for diabetes in children.

One and a half year later, I had the great opportunity to start this PhD programme that allowed me to study TRAIL in 3 paediatric cohorts with type 1 diabetes, from Naples to Cambridge passing through Trieste, and which is now coming to an end.

The aim of the project was to define the values of circulating levels of TRAIL at the onset and during the progression of type 1 diabetes in affected children, because – in spite of all data in animal or in human cell models – no studies had yet fully investigated TRAIL levels in children with type 1 diabetes.

The research was performed under the supervision of professor Alessandro Ventura (University of Trieste) and was in part supported by a grant from Italian Ministry of Health (Bando 2010 Giovani Ricercatori, code GR-2010-2310832).

ABBREVIATIONS

A1c	glycosylated haemoglobin
AICD	activation-induced cell death
Akt	protein kinase B
ALPS	autoimmune lymphoproliferative syndrome
Apo2L	Apo2 ligand
BMI	body mass index
CRP	C-reactive protein
DcR	decoy receptor
DD	death domain
DISC	death-inducing signalling complex
DKA	diabetic ketoacidosis
DR	death receptor
ERK	extracellular-signal-regulated kinases
FADD	Fas associated death domain
FasL	Fas ligand
FMD	flow-mediated endothelium-dependent arterial dilatation
GADA	glutamic acid decarboxylase antibodies
hsCRP	highly sensitive C-reactive protein
IA2A	insulinoma-associated-2 antibodies
IAA	insulin autoantibodies
ICA	islet cell antibodies
IL-2	interleukin-2
IκK	inhibitor of κB kinase
JNK	c-Jun N-terminal protein kinase
MAPKs	mitogen-activated protein kinases
MMP	matrix-metalloproteinase
NF	nuclear factor
NK	natural killer
NOD	non-obese diabetic
OPG	osteoprotegerin
PARAs	proapoptotic receptor agonists
PI3K	phosphatidyl inositol 3-Kinase
RIP	receptor interacting protein
sCD25	soluble CD25
sTRAIL	serum TRAIL
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TIMP-1	tissue inhibitor of metalloproteinase-1
TNF	tumour necrosis factor
TRADD	TNF-receptor-associated death domain
TRAF	TNF receptor-associated factor-2
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory
ZnT8A	zinc transporter-8 antibodies

ABSTRACT

Experimental evidence in animal models suggests that TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, might play an important role in type 1 diabetes (T1D). No studies had fully evaluated TRAIL levels in children affected by T1D.

Study I – *What happens to TRAIL levels in children with T1D?*

Retrospective study on 507 pediatric subjects consisting of patients diagnosed with T1D at onset (n = 167) or at later time points after diagnosis (n = 220), healthy individuals (n = 98, considered as controls), and healthy subjects positive to autoantibodies against β-cells (n = 22).

Study II – *What happens to TRAIL levels at T1D onset and/or during diabetic ketoacidosis (DKA)? And what thereafter?*

Prospective study on a cohort of 11 pediatric subjects admitted for T1D onset or secondary DKA at the Emergency Department. A total of 80 blood samples were collected at admission (serial samples until stabilization), before hospital discharge and every 6 months during the clinical follow-up up to 18 months.

Study III – *What happens to TRAIL levels in long-standing T1D? Are TRAIL levels correlated with markers of residual β-cell mass, inflammation or autoimmunity?*

Retrospective cohort study on 232 young people with long-standing T1D (median 5.5 years); 219 patients had 2 available stored non-fasting serum samples collected (median time interval between visits 1.2 years), with a total of 451 samples available.

Conclusions

1. TRAIL in type 1 diabetes

- TRAIL levels are significantly reduced in children with T1D compared to unaffected individuals
- a significant seasonal pattern of TRAIL was observed (with the lower levels during summer and higher during spring) which may have some impact on the seasonal variation in the initial presentation of T1D

2. TRAIL and autoimmunity

- there are no differences in TRAIL levels between healthy subjects positive to autoantibodies against β-cells and controls
- there are no differences in TRAIL levels between T1D patients with and without islet-specific autoantibodies
- there are no differences in TRAIL levels between T1D patients with or without other concomitant autoimmune diseases
- TRAIL levels are associated with sCD25 (IL-2RA), a known biomarker for immune activation, in long-standing T1D, both as single values and as changes over time

3. TRAIL at the onset of type 1 diabetes

- the lowest levels of TRAIL are observed in patients at the onset of disease
- among T1D patients at onset, the lowest levels of TRAIL are observed in patients with DKA
- a worst metabolic status (documented by HCO_3, BE, pH and CO_2) is associated with lower TRAIL levels
- TRAIL levels increase rapidly after short-standing insulin treatment has been established during the first hours after T1D onset and/or DKA
- there is no association between TRAIL and C-peptide or A1c or insulin daily requirement at T1D onset

4. TRAIL in long-lasting type 1 diabetes

- reduction in TRAIL levels with respect to healthy controls persisted also in patients analyzed >1 year from diagnosis
- TRAIL levels significantly increase from hospital discharge to the 6-month follow-up, and then maintain without significant modulations up to 18 months after onset
- the levels of TRAIL negatively correlate with the insulin requirement up to 21 months of follow-up
- TRAIL levels decrease with T1D duration in long-standing disease
- there is no association between TRAIL and C-peptide or CRP in long-lasting T1D

Key words: TRAIL – type 1 diabetes mellitus - ketoacidosis – autoimmunity – insulin requirement – metabolic status – sCD25

RIASSUNTO IN LINGUA ITALIANA

Evidenze sperimentali in modelli animali suggeriscono che il TNF-related apoptosis-inducing ligand (TRAIL), un membro della superfamiglia TNF, può giocare un ruolo importante nel diabete di tipo 1 (T1D). Nessuno studio aveva indagato in maniera approfondita i livelli di TRAIL nei bambini affetti da T1D.

Studio I – *Cosa succede ai livelli di TRAIL nei bambini con T1D?*

Studio retrospettivo su 507 soggetti pediatrici, consistenti in pazienti diagnosticati con T1D all'esordio (n = 167) o in un momento successivo dopo la diagnosi (n = 220), individui sani (n = 98, considerati come controlli), e soggetti sani positivi agli anticorpi diretti contro le β-cellule (n = 22).

Studio II – *Cosa succede ai livelli di TRAIL all'esordio di T1D e/o durante la chetoacidosi diabetica (DKA)? E cosa accade dopo?*

Studio prospettico su una coorte di 11 soggetti pediatrici ricoverati per esordio di T1D o DKA secondaria presso il Pronto Soccorso. Un totale di 80 campioni di sangue sono stati raccolti al momento del ricovero (campioni seriali fino alla stabilizzazione), prima della dimissione e ogni 6 mesi durante il follow-up clinico fino a 18 mesi.

Studio III – *Cosa succede ai livelli di TRAIL nel T1D di lunga durata? I valori di TRAIL sono correlati con marker di massa β-cellulare residua, infiammazione o autoimmunità?*

Studio retrospettivo di coorte su 232 giovani soggetti con T1D di lunga durata (mediana 5,5 anni); 219 pazienti avevano 2 campioni di siero disponibili, raccolti non a digiuno (mediana del tempo intercorso: 1,2 anni), per un totale di 438 campioni disponibili.

Conclusioni

1. TRAIL nel diabete di tipo 1

- i livelli di TRAIL sono significativamente ridotti nei bambini con T1D rispetto ai soggetti non affetti
- è stato riscontrato un significativo pattern stagionale di TRAIL (con i valori più bassi durante l'estate e i più alti durante la primavera) che potrebbe avere un impatto sulle variazioni stagionali nella presentazione iniziale del T1D

2. TRAIL e autoimmunità

- non ci sono differenze nei livelli di TRAIL tra soggetti sani con anticorpi diretti contro le β-cellule e i controlli
- non ci sono differenze nei livelli di TRAIL tra i pazienti con T1D con o senza gli anticorpi diretti contro le β-cellule
- non ci sono differenze nei livelli di TRAIL tra i pazienti con T1D con o senza altre malattie autoimmuni concomitanti
- i livelli di TRAIL sono associati con sCD25 (IL-2RA), un noto biomarker di attivazione immune, nel T1D di lunga durata, sia come singola determinazione che come modifica nel tempo

3. TRAIL all'esordio del diabete di tipo 1

- i valori più bassi di TRAIL sono osservati nei pazienti all'esordio di malattia
- tra i pazienti con T1D all'esordio, i valori più bassi di TRAIL si osservano nei pazienti con DKA
- uno status metabolico peggiore (documentato da HCO_3, BE, pH e CO_2) è associato con valori più bassi di TRAIL
- i livelli di TRAIL aumentano rapidamente subito dopo l'avvio di terapia insulinica nelle prime ore dopo l'esordio del T1D e/o la DKA
- non c'è associazione fra TRAIL e il C-peptide o l'HbA1c o fabbisogno insulinico all'esordio del T1D

4. TRAIL nel diabete di tipo 1 di lunga durata

- la riduzione nei livelli di TRAIL rispetto ai controlli sani persiste anche nei pazienti analizzati dopo 1 anno dalla diagnosi
- i livelli di TRAIL aumentano significativamente dalla dimissione dall'ospedale al follow-up 6 mesi dopo; dopo rimangono stabili senza modulazioni significative fino a 18 mesi dopo l'esordio
- i livelli di TRAIL correlano negativamente con il fabbisogno insulinico fino a 21 mesi di follow-up
- i livelli di TRAIL si riducono col tempo nel T1D di lunga durata
- non c'è associazione fra TRAIL e il C-peptide o la PCR nel T1D di lunga durata

Parole chiave: TRAIL – diabete mellito di tipo 1 - chetoacidosi – autoimmunità – fabbisogno insulinico – stato metabolico – sCD25

CHAPTER 1

GENERAL INTRODUCTION

1. THE LONG HISTORY OF DIABETES MELLITUS

The story of diabetes mellitus is a remarkable chronicle covering more than 3,500 years of medical history[1].

A disease characterised by the "*too great emptying of urine"* finds its place in antiquity through **Egyptian manuscripts** dating back to 1500 B.C. Indian physicians called it *madhu-meha* ("honey urine") because it attracted ants. The ancient Indian physicians **Sushruta** and **Charaka** were able to identify the two types, later to be named type 1 and type 2 diabetes[2].

The term *diabetes* ("siphon" in Greek) was introduced by **Aretaeus of Cappadocia** (81-138 A.D.): *"diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine..."*. It was in 1675 that **Thomas Willis** added the word *mellitus* ("sweet like honey" in Latin) because of the taste of the urine. However, the anatomical localization and the metabolic alteration causing diabetes remained a mystery: in the 18th century **Morgagni** (1761) wrote that diabetes was a fatal disease, the location of which was impossible to establish.

The relationship between diabetes and the pancreas was first clearly demonstrated by **von Mering and Minkowski** (1889)[3], and **Hedon** (1893) completed the postulate of an internal secretion by demonstrating that pancreas transplantation to pancreatomized dogs prevented the development of diabetes. The origin of a pancreatic factor that controlled glucose homeostasis remained to be determined.

Following the discovery of the pancreatic islet by **Langerhans** in 1869, **Laguesse** (1893) suggested that the pancreatic islets were a possible source of an internal secretion. A likely connection to diabetes was evidenced by **Dieckhoff**'s observations in 1894 that the pancreas of diabetic patients had a greatly diminished number of pancreatic islets[4].

Technical advancements, particularly in techniques of microscopy and histology, allowed **Lane** (1907) and **Bensley** (1911) to describe the pancreatic α- and β-cells, and **De Mayer** (1909) proposed insulin as the factor from the pancreatic islets that controlled blood sugar. A search for insulin was undertaken by many workers but was greatly hindered by the extensive proteolytic activity in pancreatic extracts and the specific requirements to solubilise insulin.

It was not until 1921 that **Banting** and **Best** successfully prepared pancreatic extract containing sufficient amounts of biologically active insulin[5].

The discovery of insulin had a dramatic effect on the therapy of diabetes: it was now possible to keep patients alive. Treatment during the period preceding the discovery of insulin was characterized by a starvation diet or undernutrition, with the patients allowed to receive very few calories each day. Before 1922 the life expectancy of a child or young adult with diabetes was 1 year from diagnosis. By 1924, the life expectancy had risen to 7 to 8 years, and it improved rapidly with increased knowledge of insulin action and better training of physicians and nurses. Insulin had a marked social impact; it was long thought to be the cure for diabetes. However, almost 100 years of insulin therapy have proved the hormone only maintains survival in a chronic disorder associated with a risk of death that is twice as high as controls even in presence of a very good glycaemic control[6].

2. TYPE 1 DIABETES MELLITUS

Diabetes mellitus refers to a condition of hyperglycaemia that can be further classified into **type 1**, primarily due to a lack of insulin, or **type 2**, primarily due to peripheral insulin resistance.

Type 1 diabetes (T1D) is one of the **most common endocrine and metabolic conditions in childhood**, showing an **increasing incidence rate** of about 3% per year during the last two decades. During the period 1990–2003 the incidence rate in Italy was 12.26 per 100,000 person-years, with an increasing temporal trend of 2.94% per year[7]. Maintaining this trend, a doubling of new cases of T1D in children is expected between 2005 and 2020, with increasing burden for the families and the health care system[8].

T1D is generally thought to be precipitated by an **immune-associated**, if not directly immune-mediated, **destruction of insulin-producing pancreatic β-cells**[9].

The concept that T1D **pathogenesis** is **immune-mediated** is half a century old, dating back to the Sixties, when Burnet and Mackay included T1D among other autoimmune diseases (*"a condition in which structural or functional damage is produced by the action of immunologically competent cells or antibodies against normal components of the body"* arising by *"the emergence of forbidden clones of T lymphocytes"*)[10]. Two years later, Gepts found that insulitis was the key pancreatic morphological feature in recent-onset T1D and this led him to suggest that the immune system is involved in the pathogenesis of the disease[11]. Then, in 1974 Nerup demonstrated that T1D is a cell-mediated autoimmune disease by incubating peripheral blood mononuclear cells of diabetic patients with porcine islets material[12].

In terms of potential pathogenic mechanisms, **$CD8^+$ T cells** are the most predominant population within the insulitis lesion, followed by (in declining order) **macrophages** ($CD68^+$), **$CD4^+$ T cells**, **B lymphocytes** ($CD20^+$), and

plasma cells ($CD138^+$)[13]. Surprisingly, $FOXP3^+$ cells (i.e., regulatory T cells) and natural killer (NK) cells are rare in this lesion.

A key distinguishing feature of T1D is the **presence of autoantibodies against β-cell autoantigens**. More than 90% of individuals with newly diagnosed T1D have one or more of the following autoantibodies at disease onset[14]: those reactive to islet cell (ICA), insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A), and zinc transporter 8 (ZnT8A)[15]. These autoantibodies can appear as early as 6 months of age, with a peak incidence before 2 years of age in genetically susceptible individuals[16]; thus, they are **present months to years before symptomatic onset**. In addition to having diagnostic value in T1D, autoantibodies can help identify **people with an increased risk for developing the disease**, through detection in first-degree relatives or in the general population. IAA concentration correlates with the rate of progression to overt type 1 diabetes in children followed from birth[17,18]. This finding, combined with an extensive series of independent investigations in humans and in rodent models of T1D, support the growing notion that proinsulin is a key autoantigen in the disease[19], a concept that might partly explain the selective β-cell loss in T1D.

Another supportive evidence for the autoimmune pathogenesis of T1D comes from the **susceptibility** of these individuals **to other autoimmune conditions**, including Hashimoto's thyroiditis, Graves' disease, Addison's disease, coeliac disease, myasthenia gravis, and vitiligo[20–23].

Inflammation might contribute to early induction and amplification of the immune assault against pancreatic β-cells and, at later stages, to the stabilization and maintenance of insulitis. Inflammatory mediators probably contribute to the suppression of β-cell function and subsequent apoptosis; they may also inhibit or stimulate β-cell regeneration and might cause peripheral insulin resistance. The different effects of inflammation take place in different phases of the course of T1D, and should be considered in the context of a 'dialog' between invading immune cells and the target β-cells. This dialog is

mediated both by cytokines and chemokines that are released by β-cells and immune cells, and by putative, immunogenic signals that are delivered by dying β-cells[24].

In the 1986 Eisenbarth proposed the **current model for the development of the immune form of T1D**[25] and although our understanding has progressed significantly since then, the basic aspects of this model remain pertinent[9,26,27] *(Figure 1)*.

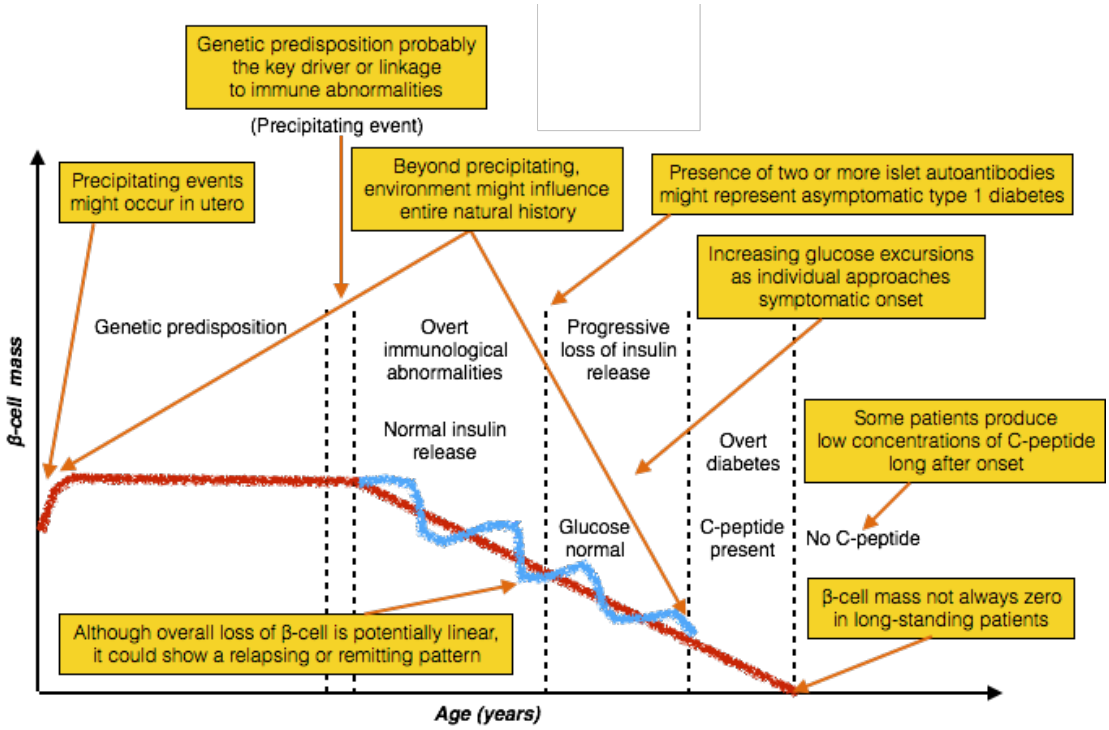


Figure 1 - *The natural history of type 1 diabetes according to Eisenbarth's model (originally proposed in 1986, then updated in 2001 and 2014)[9]. Latest additions and conjectures based on recent knowledge gains are shown in yellow boxes.*

This model postulates that everyone is born with a degree of susceptibility to develop T1D: for some this susceptibility is high, for others very low. Susceptibility is largely inherited, residing predominantly in the HLA genotypes DR and DQ, and to a lesser extent in a host of other genetic loci termed *IDDM* (insulin-dependent diabetes mellitus) susceptibility genes. The HLA locus is thought to confer about 50% of the genetic susceptibility, roughly 15% from two other genes – *insulin-VNTR* (*IDDM2*) and *CTLA-4* (*IDDM12*) – with minor contributions from the other *IDDM* genes[28–31]. Both high risk (i.e., *DR3/4, DQA1*0301-DQB1*0302,* and *DQA1*0501-DQB1*0201*) and protective HLA

haplotypes (i.e., *DQA1*0102-DQB1*0602,* associated with diabetes resistance, and DR molecules such as *DRB1*1401*, associated with protection from diabetes) have been identified[28]. These susceptibility genes are thought to be important regulators of the immune response. Other genes associated with either rare syndromes including diabetes (i.e., *AIRE* and *Foxp3*) or other autoimmune conditions (i.e., *PTPN22*) might also provide important insights into the immune pathogenesis of T1D.

In the **beginning** the **autoimmune destruction** of the insulin-producing β-cells in the pancreas is **asymptomatic.** This insidious process may evolve over a period of years (2-8 years)[26].

Continuing destruction of cells leads to progressive loss of insulin-secretory reserve with, in order, loss of first phase insulin secretion in response to an intravenous glucose tolerance test *(Figure 2)*, then to clinical diabetes when insulin secretion falls below a critical amount, and finally, in most but not all those with T1D, to a state of absolute insulin deficiency[25,30].

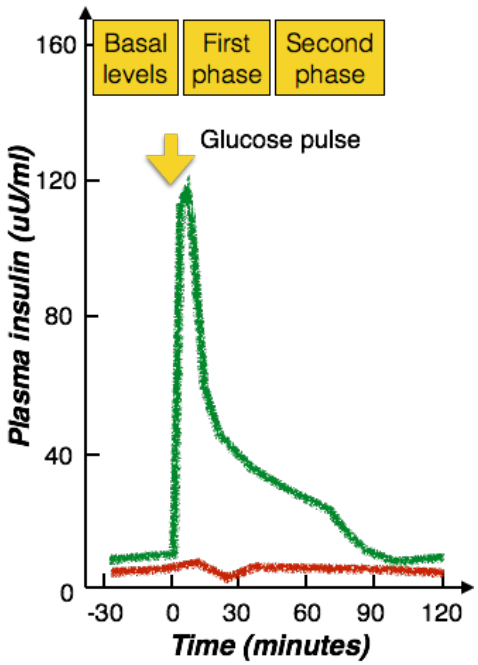


Figure 2 - *Schematic representation of insulin release in response to an intravenous injection of glucose. The green line is the normal response, the red line is the response in a patient with T1D*[32]

Prediction of diabetes in relatives of a person with T1D and in the general population can be determined by risk assessment that includes HLA genotyping and measurement of autoantibodies combined with test of β-cell function[33,34]. Autoantibodies restricted to a single antigen have little prognostic value but an immune response that has spread to multiple antigens and is stable over time is highly predictive[35,36]. Individuals who have multiple islet autoantibodies are destined to develop T1D.

The **latency period** offers an **opportunity to intervene** but oral insulin[34], intranasal insulin[37], parenteral insulin[38] and nicotinamide[39] have failed to arrest or retard the diabetes disease process. We can predict the development of T1D but **do not yet have an effective preventive therapy**[40]; whether screening should be performed outside the context of clinical studies is controversial[41].

Although it is often stated that symptoms occur when 90-95% of β-cells are lost, **diagnosis** of T1D can **occur when roughly two-thirds of the islets are devoid of insulin-producing cells**[13,42].

The early development of T1D involves progressive insulin deficiency, leading to weeks of symptomatic hyperglycaemia with **polyuria, polydipsia and weight loss**. If left untreated, the combination of insulin deficiency and stress (mediated through increased circulating levels of counter-regulatory hormones, including cortisol, catecholamines, growth hormone and glucagon) leads to lipolysis. Hepatic metabolism of free fatty acids as an alternative energy source (i.e., ketogenesis) results in accumulation of acidic intermediate and end metabolites (i.e., ketones and ketoacids). **Diabetic ketoacidosis** (DKA) then occurs in the presence of severe insulinopenia and is characterized by hyperglycaemia, acidosis and ketosis. Diagnosis of DKA has been traditionally made with a combination of hyperglycaemia (blood glucose >200 mg/dL), venous pH less than 7.3 and bicarbonate levels less than 15 mmol/L[43]. DKA at initial presentation is common, but rates vary strikingly between countries, ranging from approximately 15% to 70% in Europe and North America[44] (32.9% in Italy, 6.6% of the severe form[45]). An inverse correlation between DKA frequency at diagnosis and the regional background incidence of T1D has been

described[43]. The key individual factors associated with greater risk of DKA are being less than 2 years old at presentation, being incorrectly diagnosed or having treatment delayed, belonging to an ethnic minority, lower socio-economic status, lack of health insurance in the United States, lower parental education, lower body mass index (BMI) and preceding infection[46–48].

DKA remains the **most common cause of mortality in T1D**. The most devastating consequence of DKA is cerebral oedema with an incidence of 0.5% to 0.9%[49,50]. The mortality rate from cerebral oedema is 21% to 24%, and morbidity from serious neurologic sequelae occurs in 15% to 35% of cases[48,51]. Furthermore, DKA has a long-term impact on cognitive function, including a fall in intelligence quotient and persistent loss of short-term memory[49].

The **"honeymoon"** or **remission period** – characterized by an improvement in residual β-cell function with increasing concentrations of circulating C-peptide and a lower requirement for exogenous insulin[52–56] – takes place soon after clinical diagnosis and initiation of insulin therapy when some endogenous insulin secretion is restored to exhausted but not yet destroyed cells, and when insulin resistance associated with initial hyperglycaemia is lessened[57,58]. The phenomenon is thought to be due to an **increase in insulin sensitivity**[58,59] and an **alleviation of the destructive process** in the islets of Langerhans. The insulin treatment reduces hyperglycaemia and the demand on the β-cells to secrete insulin, β-cell rest[60], which, as exemplified by *in vitro* experiments, lowers their autoantigen expression[61,62] and improves the resistance against destruction with β-cell toxins[63,64]. In general, **β-cells are destroyed more rapidly when onset of clinical diabetes takes place at a young age**, when there is also less likelihood of a long remission period *(Figure 3)*. Thus, older individuals are more likely to respond soon after diagnosis to immune interventions aimed at preserving residual insulin secretion[27].

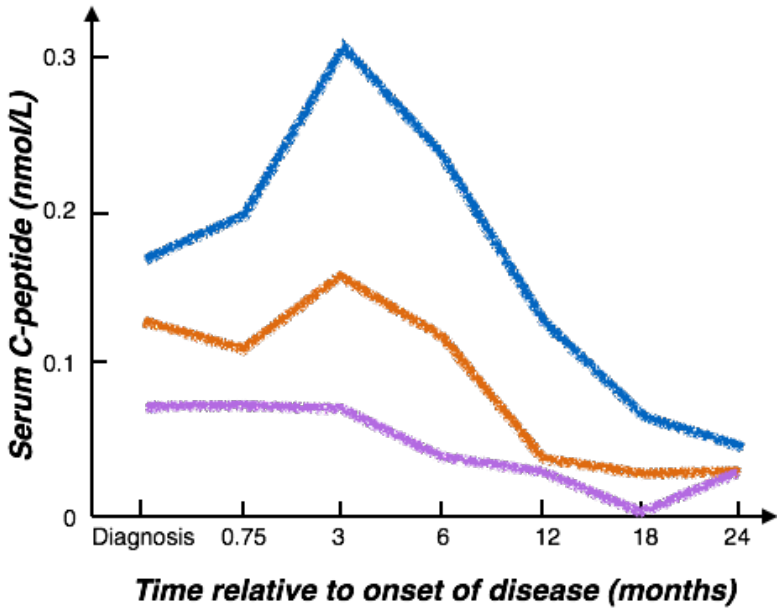


Figure 3 - *Younger children more rapidly lose endogenous insulin production, as evidenced by plasma C-peptide concentrations from the time of diagnosis of T1D. Data are stratified by age of onset: 5–14.9 years old (blue), 2–4.9 years old (orange) and <2 years old (purple). Toddlers have the lowest plasma C-peptide concentrations at diagnosis. The temporary partial remission experienced by older children is readily apparent and is notably absent in toddlers.*[65]

Among individuals who have had T1D for more than 5 years, most of the remaining islets are insulin deficient, containing a normal complement of other hormone-secreting cells (i.e., α-cells that secrete glucagon, δ-cells that secrete somatostatin, and PP-cells that secrete pancreatic polypeptide)[13], thus T1D involves a selective loss of β-cells. Recent data suggest that although most patients with long-standing T1D have few β-cells, if any, there is evidence for **β-cell regeneration in infants and very young children** (but not in adolescents or adults)[66,67].

Highly sensitive and specific radioimmunoassay for the proinsulin C-peptide permit studies of **residual β-cell function** despite a patient being treated with insulin[68]. The use of the C-peptide is based on the observation that insulin and C-peptide are released in equimolar amounts from the cells. Fasting C-peptide is reduced, often to a level below the normal range *(Figure 4)*.

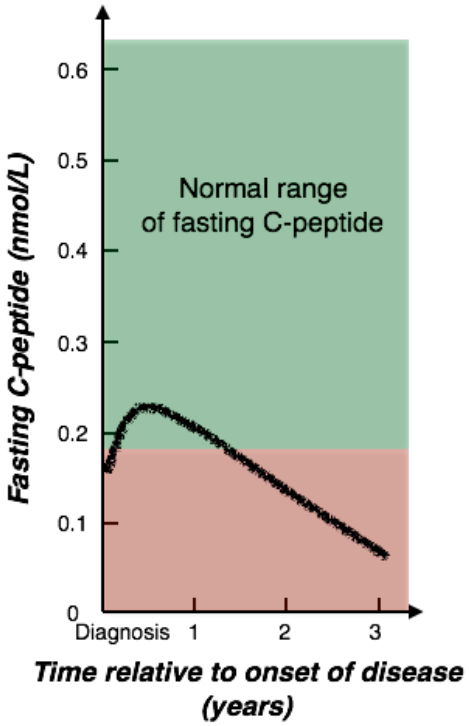


Figure 4 - *Schematic representation of fasting plasma C-peptide range in healthy individuals. The solid line is the average fasting C-peptide level for insulin-dependent diabetic patients in relation to duration of the disease in years*[32].

The initial insulin treatment appears to increase the level of fasting C-peptide during the first 3-6 months after diagnosis. After that, C-peptide levels continuously decrease, reaching values below the detection limit of the assay (about 0.05 pmol/ml) after 3 to 4 years of T1D[69–71]. The extent to which fasting or stimulated levels of C-peptide reflect the residual mass of pancreatic β cells remains to be clarified. It is clear that, within hours, removal of exogenous insulin hampers the ability of a T1D patient to control blood sugar. However, it is not clear whether the residual amount of C-peptide and its fluctuation following dietary control or intensified insulin therapy reflect changes in the number or residual β-cells or in the function of those remaining β-cells.

There is good evidence that the **residual 10% of β-cell function has clinical benefit at the onset of symptoms**. The Diabetes Control and Complications Trial (DCCT) identified a “virtuous circle” whereby residual insulin secretion resulted in better glucose control with less hypoglycaemia and slower progression to vascular complications[72]. Much investigation has been directed at arresting the disease process during the evolution of the disease and at

presentation[40]. The goal is to halt β-cell destruction, thereby lessening the severity of clinical manifestations and disease progression[41].

In the last years, interest in reversal of T1D diabetes has grown[73]. Currently, there are **no approved agents to stop the autoimmune destruction of β-cells after diagnosis of T1D**[9]. In addition to **preserving production of C-peptide**, a key goal is to **induce immune tolerance against β-cells** and thereby halt autoimmune destruction. Most approaches involve provision of self-antigen (i.e., vaccination with specific islet-cell proteins, such as insulin or GAD) or immune suppression. Disappointingly, after promising phase 1–2 trials, phase 3 trials did not meet primary endpoints. Other phase 2 studies of immune modulators showed evidence of therapeutic efficacy in settings of recent-onset T1D; however, even with continued use, most did not show durable effects *(Table 1)*[9].

Table 1 - *Agents assessed as immunomodulatory therapy to reverse T1D*[9]

Agent	Study phase; year	Main findings
Insulin APL *NBI-6042*	Phase 2; 2009	No change in metabolic response (i.e., C-peptide preservation)[74]
Anti-CD20 *Rituximab*	Phase 2; 2011	Preservation of C-peptide concentrations at 1 year, but no diff erence from placebo at 2 years[75]
Anti-CD3 *Teplizumab*	Phase 3; 2011	Although phase 2 studies showed preservation of C-peptide concentrations, phase 3 trials (Protégé study)[76] showed no change in metabolic respons and the study stopped early
CTLA4 - immunoglobulin fusion protein *Abatacept*	Phase 2; 2011	T-cell co-stimulatory modulation slowed reduction in β-cell function over 2 years, although preservation of C-peptide was seen for 9.6 months[77]
Anti-CD3 *Otelixizumab*	Phase 3; 2011	Although phase 2 studies showed preservation of C-peptide concentrations, a phase 3 trial showed no change in metabolic response[78]
GAD65 protein *Diamyd*	Phase 3; 2012	Phase 2 studies reported preserved C-peptide concentration, with no improvements in insulin needs. Two phase 3 trials did not meet endpoints[79,80]
HSP60 *DiaPep277*	Phase 3; 2012	Phase 2 trials suggested increased C-peptide concentrations; a phase 3 trial noted C-peptide preservation at 1 year, but only in adults (age 16-45 years) with T1D[81]

These results imply that single-agent immunosuppression alone might be insufficient to completely control the autoimmune destruction of β cells, or that more specific and targeted therapies are needed.

Combination therapies that target several pathogenic pathways and improve β-cell viability might be needed to preserve endogenous insulin production in patients with T1D. Additionally, testing agents that target inflammation (i.e., anakinra [interleukin-1 receptor antagonist] and canakinumab [anti-interleukin-1b compound]), alone or in combination could prove beneficial[82].

3. TRAIL

Tumor necrosis factor [TNF]-Related Apoptosis-Inducing Ligand (TRAIL) (also known as Apo2 ligand or Apo2L) is a member of the TNF superfamily of proteins, whose best characterized function is the **induction of apoptosis in tumour, infected, or transformed cells through activation of specific receptors**[83].

Apoptosis is a process leading to cell death, whereby unrequired cells can be eliminated in order to safeguard multicellular organism health. There are two ways of signalling leading to apoptosis. One is called *"intrinsic pathway"*, because it is triggered by an intracellular signal, such as DNA damage, while the other is called *"extrinsic pathway"*, because it is triggered by an extracellular signal, which usually derives from cytotoxic cells of the immune system *(Figure 5)*[84].

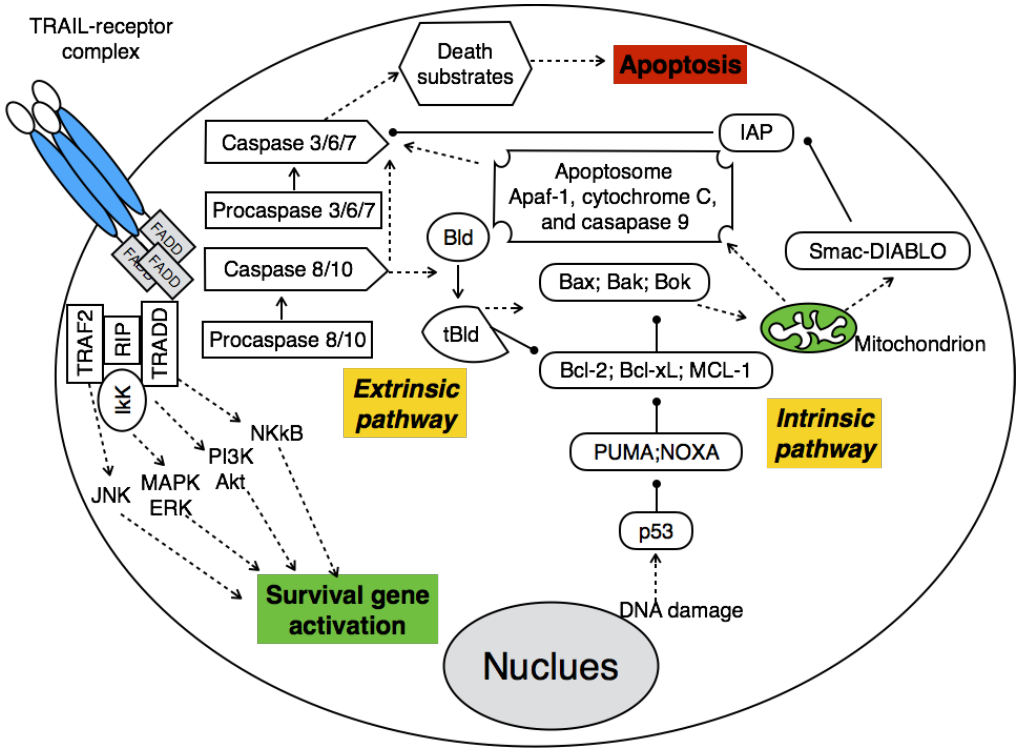


Figure 5 - *TRAIL-receptor mediated signalling pathways. By binding its receptor TRAIL initiates cell death (apoptosis) via either intrinsic (mitochondria) or extrinsic pathway and/or induces the activation of survival genes resulting in cell proliferation/migration and inhibition of apoptosis.*[83]

In particular, the **extrinsic pathway** is activated upon the **binding** of specific **proapoptotic ligands**, namely, **FasL/CD95L** and **TNF-α**, to their **transmembrane receptors**. This stimulates the trimerization of the transmembrane receptors and the formation of the death-inducing signalling complex (DISC), based on the recruitment of Fas associated death domain (FADD). Subsequently, FADD recruits both caspase-8 and caspase-10, which undergo autoactivation by proteolytic cleavage and which in turn activate caspase-3, caspase-6, and caspase-7, eventually executing the apoptotic program *(Figure 5)*.

TRAIL was independently identified by two different groups as the **third pro-apoptotic ligand that triggers the extrinsic pathway**[85,86]. The name was chosen for the high homology of this protein to other TNF family members (the percentage of identity with FasL/CD95L and TNF-α is in fact 28% and 23%, respectively). The gene encoding for TRAIL is located on chromosome 3 at position 3q26. TRAIL gene locus spans approximately 20 kb and it has five exonic segments and four introns.

In humans, TRAIL is expressed as a type II transmembrane protein of 281 amino acids and it is composed of four parts: (1) an extracellular TNF-like domain; (2) an extracellular stalk; (3) a transmembrane helix; (4) a small cytoplasmatic domain.

There is potential cleavage site in the extracellular domain at aminoacid position 114. TRAIL can be **proteolytically cleaved** by cysteine proteases and matrix-metalloproteinase(MMP)-2 to produce a **soluble form** of 24 kDa **with biological activity**. It is at this stage that the cysteine residue at position 230 (Cys230) allows TRAIL to interact and assemble with other two molecules of TRAIL forming a **trimeric ligand**. These homotrimers are proapoptotic agonists that bind to their specific receptors *(Figure 6)*.

Unlike the other members of the TNF superfamily, TRAIL binds to a complex system of receptors with distinct affinities and different signaling outcomes.

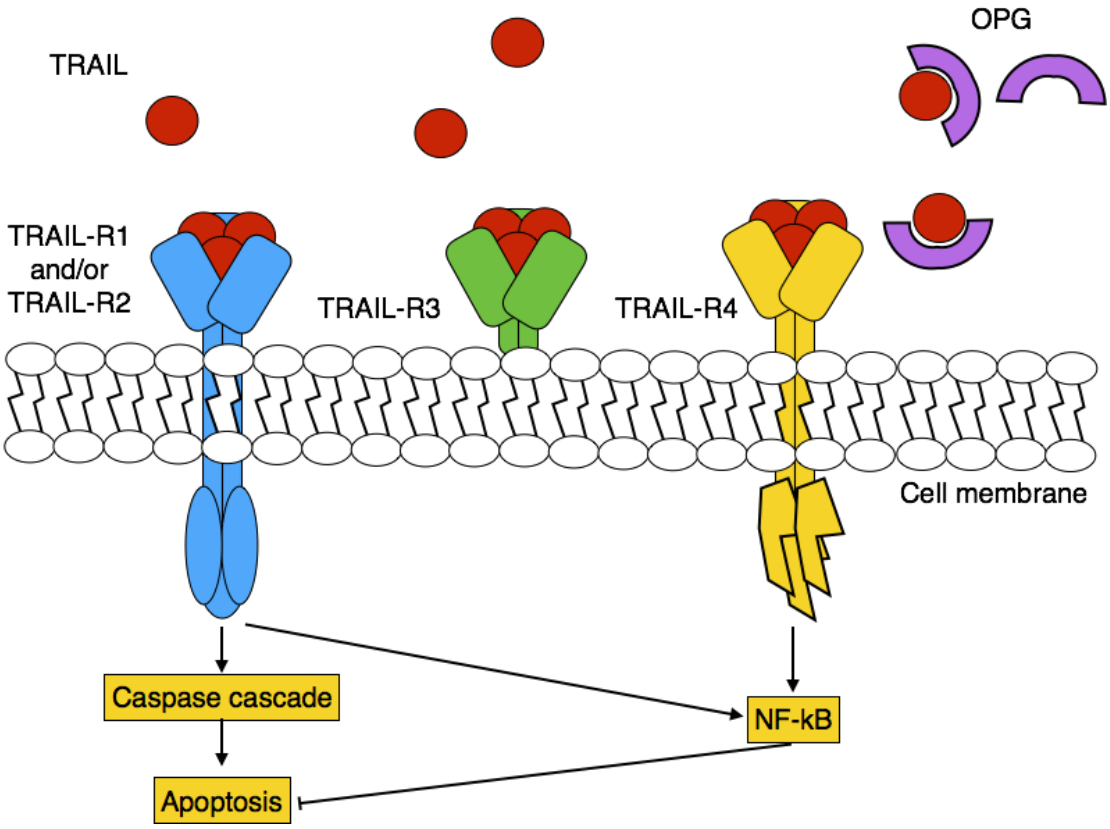


Figure 6 - *Schematic representation of TRAIL receptors. The binding of trimeric soluble TRAIL with death receptors TRAIL-R1 and TRAIL-R2 can induce either the activation of the caspase cascade, promoting cellular apoptosis, or the NF-κB signaling pathways. TRAIL also binds other 2 transmembrane receptors (TRAIL-R3 and TRAIL-R4) and one soluble receptor (OPG). TRAIL-R4, which has a truncated death domain, is not able to trigger the apoptotic signal, but may activate NF-κB pathway.*[87]

Four membrane receptors (not only death receptors but also decoy receptors) and a soluble receptor are known in humans, while mice express a unique apoptosis-inducing receptor for TRAIL.

The human receptors for TRAIL are:

- 2 **death receptors** (DR) which are both type I transmembrane proteins containing an intracellular death domain (DD) that classically stimulates apoptosis upon TRAIL binding by activating both caspases and nuclear factor (NF)-κB pathways:
    - **TRAIL receptor 1 (TRAIL-R1)** or death receptor 4 (DR4) or TNFRSF10A [88]
    - **TRAIL receptor 2 (TRAIL-R2)** or death receptor 5 (DR5) or TNFRSF10B or TRICK2 or KILLER[89,90]

- 2 **decoy receptors** (DcR) which are transmembrane receptors that differ from DR in that their cytoplasmatic domain lacks an intact DD and apoptosis-inducing capability; they are not capable of activating caspase cascade, but may activate NF-κB and block apoptosis; they have been proposed to protect normal cells from apoptosis:
    - **TRAIL receptor 3 (TRAIL-R3)** or decoy receptor 1 (DcR1) or TRID or TNFRSF10C [91]
    - **TRAIL receptor 4 (TRAIL-R4)** or decoy receptor 2 (DcR2) or TNFRSF10D [92]
- 1 **soluble receptor** that lacks both transmembrane and cytoplasmatic residues; it seems to inhibit TRAIL-induced apoptosis by competitive inhibition of TRAIL-binding to the death receptors TRAIL-R1 and TRAIL-R2:
    - **osteoprotegerin (OPG)**[93]

Depending on time, dose and location of TRAIL expression and engagement of special receptors, it may exert a beneficial as well as a destructive potential[94].

The first and best characterized function of TRAIL was its ability to **induce apoptosis in transformed cells**, such as **malignant cells**. Studies on TRAIL knockout mice have in fact demonstrated that mice without TRAIL are viable and fertile but more susceptible to tumour metastases, indicating that **TRAIL regulates immune surveillance and host defence against tumour initiation and progression**[95,96]. In particular, TRAIL seems to mediate the ability of NK cells and cytotoxic T lymphocytes to block tumour growth and metastasis development[97,98]. It should be noted that TRAIL-dependent killing by NK cells is believed to **protect the organism also from infection**[99]. Interestingly, one of the unique aspects of TRAIL, as compared to the other pro-apoptotic receptor agonists (PARAs)[100,101], is that TRAIL has the ability to **induce apoptosis preferentially in transformed cells**, such as tumour or infected cells, **while it would spare most normal cells,** except thymocytes, neural cells and human hepatocytes[102].

Another intriguing aspect of TRAIL is that it **can apparently mediate also non apoptotic signalling**. When TRAIL-R1 and TRAIL-R2 bind to TRAIL homotrimers they can stimulate not only pro-apoptotic pathways, but also pro-survival pathways, such as NF-κB, ERK1/ERK2, and Akt[103,104] *(Figure 5)*. In particular the pro-survival signalling complex would still rely on DISC assembly, but instead of caspase activation, it would depend on the recruitment of TNF-receptor-associated death domain (TRADD), TNF receptor-associated factor-2 (TRAF2), receptor interacting protein (RIP), and the inhibitor of κB kinase (IκK). Once this complex has been assembled, it would then activate NFκB, PI3K/Akt (phosphatidyl inositol 3-kinase/protein kinase B), and MAPKs (mitogen-activated protein kinases), including ERK (extracellular-signal-regulated kinases), as well as JNK (c-Jun N-terminal protein kinase) and p38, leading to survival signals *(Figure 5)*.

In regards to the **ability of TRAIL to activate diametrically opposed pathways**, it has been suggested that this may derive from **differential cell responsiveness**, rather than to the **ligand properties**. In this respect, it has been proposed that cell responsiveness to TRAIL could result from the balance between death and decoy receptors expressed on the cell surface. It is current view that this balance can be influenced by the cellular incorporation of specific intracellular proteins into “lipid rafts”. Lipid rafts are platforms consisting of a dynamic pool of cholesterol and sphingolipids, which recruit signalling molecules including cell surface receptors. Several studies have reported that there is a redistribution of TRAIL receptors and DISC components from “non-rafts” into “lipid rafts” and it is current opinion that this is what could switch either cell apoptosis or survival upon TRAIL stimulation[105,106].

However, a correlation between TRAIL receptors balance (i.e., death/decoy receptor ratio) and protection versus susceptibility to apoptosis has never been clearly demonstrated and it is still under debate.

4. TRAIL AND AUTOIMMUNITY

During the last decade it has been revealed that **TRAIL is clearly implicated not only in cancer but also in immunity**. It has been shown to exert immunosuppressive and immunoregulatory functions important for immune homeostasis, immunosurveillance and autoimmunity.

Although neither TRAIL nor TRAIL-R knockout mice develop spontaneous autoimmune diseases, TRAIL has consistently been shown to inhibit autoimmune diseases in a variety of animal models.

Animal studies have shown that genetic loss of both FasL and TRAIL resulted in a condition that resembles to human autoimmune lymphoproliferative syndrome (ALPS), which is characterized by splenomegaly and lymphadenopathy, due to an accumulation of $CD4^{-}CD8^{-}$ "double negative" T cells[107]. The fact that in this model TRAIL deficiency affected the severity of lymphocyte accumulation indicates that **TRAIL contributes to the control of peripheral lymphocyte apoptosis**, perhaps in a secondary or cooperative manner with respect to FasL. In addition, the finding that TRAIL deficiency led to ALPS2 development, which is characterized by abnormal dendritic cells accumulation[108], suggests that TRAIL helps in getting rid of immature dendritic cells, by killing them before lymph node entry.

Moreover, the observation that TRAIL-knockout mice had a severe defect in thymocyte apoptosis and were hypersensitive to both collagen-induced arthritis and streptozotocin(STZ)-induced diabetes initially suggested that **TRAIL could be critically involved in the maintenance of central tolerance by the negative selection of autoreactive thymocytes**[109]. Nevertheless, further studies have challenged this hypothesis. Cretney and colleagues could in fact not reproduce the finding that negative selection was impaired in TRAIL-knockout mice[110], nor that the adaptor protein FADD was essential for thymic negative selection[111]. Eventually, though, Corazza and colleagues reconciled these conflicting results by suggesting that in the thymus TRAIL is a response

modifier for mitochondrial apoptosis rather than a direct mechanism of thymic negative selection[112], therefore implying that **TRAIL is involved, although only indirectly, in thymic apoptosis and thymic negative selection.**

If central tolerance relies on thymic negative selection, peripheral tolerance is based on: (1) anergy induction; (2) presence of $CD4^+CD25^+$ regulatory T (Treg) cells; (3) termination of T cell immune responses, which depends on the activation-induced cell death (AICD). It is in fact through AICD or by eliminating activated immune cells that the organism prevents any potential autoimmune damage.

One of the first observations suggesting TRAIL involvement in mature lymphocyte post stimulation apoptosis was that **TRAIL induced apoptosis in T cells after sensitization with interleukin(IL)-2**[113]. Subsequently, TRAIL was also found to play a role in AICD of human peripheral blood mononuclear cells[113], which is consistent with the concept that this molecule **helps in getting rid of potentially dangerous immune cells**. Apart from AICD, TRAIL seems to regulate peripheral tolerance also by **promoting the proliferation of Treg cells**[114,115]. These are cells that play an essential role in maintaining immune tolerance, and when they are absent, such as in individuals with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), there is an enhanced susceptibility to autoimmune diseases and diabetes. In addition to that, the third way whereby TRAIL could regulate peripheral tolerance is its ability to **prevent primary T cell proliferation during the antigen-independent T cell activation**[116].

Interestingly, it seems that TRAIL affects adaptive immune cells not only by inducing cell death, but also by inhibiting their activation and expansion[117]. On one hand, it has been shown that **differentiating $CD4^+$ Th1 cells**[118] are **selectively removed by TRAIL-mediated apoptosis**[119], which explains why the frequency of $CD4^+$ Th1 cells is greater in TRAIL-knockout mice with respect to their controls[114]. Likewise, also **$CD8^+$ antigen-activated T cells, generated in the absence of $CD4^+$ T cells ("helpless" $CD8^+$ T cells), are removed by TRAIL-mediated apoptosis**[97]. On the other hand, **in $CD8^+$ T cells TRAIL can**

induce cell cycle arrest in G2/M phase (rather than apoptosis)[120]. Nevertheless, TRAIL can reduce T cell proliferation also by inhibiting calcium influx[121]. Not surprisingly, TRAIL is also involved in the **regulation of plasma cell homeostasis**, where it promotes apoptosis under specific conditions, which include the loss of both CD40 expression and NFκB activation[122]. Consistent with this, **administration of neutralizing anti-TRAIL antibodies markedly increases serum autoantibody levels** in autoimmune prone mice[123].

To complete the picture, TRAIL seems to have also **anti-inflammatory actions**. For example, Renshaw and colleagues demonstrated that neutrophil apoptosis is accelerated by leucine zipper-tagged TRAIL, which may represent a potential mechanism of neutrophil clearance at sites of inflammation[124]. Another argument in favour of TRAIL anti-inflammatory actions is what it does on atherosclerosis[125], which should in fact be considered as an inflammatory disease[126]. In *in vitro* studies, TRAIL significantly reduced leukocyte/endothelial cell adhesion by down-regulating chemokine expression[127]. In *in vivo* studies, TRAIL delivery reduced the extent of aortic atherosclerosis, possibly by inducing macrophage apoptosis[128], and consistent with this finding, DiBartolo and colleagues reported a reduction in macrophage accumulation in the atherosclerotic plaques of TRAIL-knockout mice[129].

5. TRAIL AND DIABETES

The function of TRAIL in the pathogenesis of diabetes was initially recognized in rodent models, suggesting that **TRAIL might protect against T1D**[83,87].

The potential role of TRAIL in T1D was first explored in 2003 by Lamhamedi-Cherradi and colleagues[109]: **TRAIL-deficient mice were hypersensitive to STZ-induced diabetes** and collagen-induced arthritis, and developed heightened autoimmune responses[109].

The same group examined the consequences of TRAIL blockade or TRAIL deficiency in two animal models of T1D[130]. The first animal model consisted of NOD (non obese diabetic) mice injected with a soluble TRAIL receptor (sDR5/TRAIL-R2) in order to block TRAIL functions. **TRAIL blockade** by sDR5 injection **significantly increased T1D incidence and accelerated T1D onset in NOD mice**. Moreover, in this study sDR5 injection led to a **greater islet inflammation** as well as to **enhanced cellular and humoral immune responses**, with an increase in T cells proliferation and pro-inflammatory response as well as higher levels of anti-GAD-65[130]. The second animal model consisted of normal and TRAIL-knockout mice injected with multiple low-dose STZ in order to destroy β-cells and therefore to cause diabetes. The findings in the diabetic TRAIL-knockout mice recapitulated those observed in the sDR5 treated NOD mice: both the **incidence and the degree of islet inflammation were significantly enhanced in TRAIL-deficient animals**[130].

Consistently with these observations, **adenoviral delivery of TRAIL significantly lowered diabetes incidence in NOD mice**[131]. In STZ-induced diabetic rats undergoing pancreatic islet transplantation, **viral TRAIL administration prolonged normal blood glucose levels** up to 60 days, and displayed significantly **reduced pancreatic insulitis** compared to controls[132]. Furthermore, **administration of recombinant TRAIL significantly reduced hyperglycemia** in STZ-treated mice, associated with **larger pancreatic islets**[133].

Anyhow, the molecular mechanisms whereby TRAIL blockade exacerbates T1D and, on the other side, its delivery ameliorates the natural history of the disease have only partly been clarified.

The **potential mechanisms underlying TRAIL protective effects** include interaction with the immune activation and innate immunity, such as pro-apoptotic actions on autoreactive T cells[134], stimulation of Treg cells[114], anti-inflammatory effects[135] and reduction of cytokine-mediated cytotoxicity in pancreatic islets[136].

One hypothesis is that TRAIL could **induce T cell death or inhibit their activation**. This has been partly confirmed by Mi and colleagues who showed that **TRAIL suppressed *in vitro* the proliferation of autoreactive T cells isolated from diabetic NOD mice**[134]. This effect was explained by the finding that TRAIL up-regulated p27 expression and simultaneously inhibited IL-2 production by autoreactive T cells. In this respect, it has indeed been shown that the up-regulation of p27 stops the progression of the cell cycle, preventing the progression of T cells through the G1 restriction point of the cell cycle, and therefore anergizing autorective T cells[137]. On the other hand, cell exposure to IL-2 has been shown to promote the degradation of p27 and the entry into S phase[137]; therefore **IL-2 inhibition by TRAIL could be an additional mechanism whereby it anergizes diabetogenic T cells**.

Another hypothesis is that TRAIL could **promote β-cell survival**[87]. *In vitro* experiments performed on rat insulinoma cells (INS-1), which are used as a model for the study of pancreatic β-cells, have shown that exposure to soluble TRAIL does not affect β-cell viability, and, by promoting the activation of NF-κB, upregulates the expression of the decoy receptor TRAIL-R3, which in turns **should prevent apoptosis**[138]. In this context, it is noteworthy that specific increase of TRAIL and TRAIL-R3 expression in the pancreatic islets in NOD mice, upon acceleration of T1D disease by STZ injection, has been proposed as part of a defensive strategy of the islets against the infiltrating leukocytes[132].

Kang and colleagues have further investigated TRAIL protective effects in T1D animal model, demonstrating that they could be partly attributed to the **elevation of tissue inhibitor of metalloproteinase-1** (TIMP-1) levels that follows TRAIL delivery[131]. *In vivo*, TIMP-1 elevation induced by TRAIL delivery **markedly reduced the pancreatic activity of MMP-9**, thus contributing to the preventive effect of TRAIL on T1D in NOD mice[131]. Of interest, it has been previously reported that plasma concentrations of MMP-9 are elevated in diabetic patients and that increased MMP-9 is diabetogenic as it cleaves insulin[139–141]. The *in vivo* data are further supported by *in vitro* data showing that the addition of TIMP-1 significantly **reduces INS-1 cells death**[131], consistently with a previous report showing that TIMP-1 **prevents cytokine-mediated dysfunction and cytotoxicity in pancreatic islets and β-cell**[136] *(Figure 7)*.

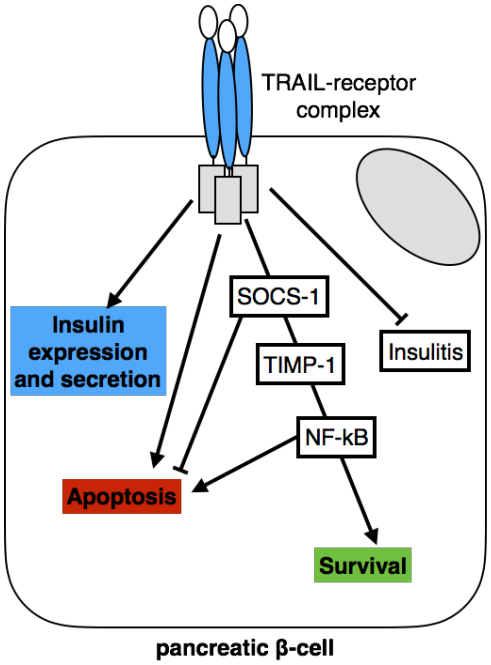


Figure 7 – *Effects of TRAIL in pancreatic β-cells. TRAIL can stimulate caspase- or NF-κB-mediated apoptosis, or survival via NF-κB, tissue inhibitor of metalloproteinase-1 (TIMP-1), and possibly suppressor of cytokine signaling-1 (SOCS-1). TRAIL not only promotes insulin production/secretion from β-cells but also prevents insulitis, in part via TIMP-1 inhibitory effects on leukocyte infiltration in the pancreas*[142].

In humans, **TRAIL expression is detected in the islets of acute-onset T1D patients** but is absent in healthy individuals[143] and this corresponded with **increased TRAIL expression in T cell lines** derived from new-onset T1D patients, upon stimulation with β-cell antigens[143], and in peripheral T cells from T1D patients[144].

6. DULANERMIN: A POTENTIAL THERAPEUTIC CHANCE

Dulanermin (AMG 951) is the **recombinant human soluble** protein corresponding to amino acids 114-281 of **TRAIL**[145]. Because of its potential antineoplastic activity, it has been studied as a PARAs in cancer therapy. Dulanermin binds to and activates death receptors (TRAIL-R1/R2), which may activate caspases and induce p53-independent apoptosis in TRAIL-R1/R2-expressing tumour cells. The pro-apoptotic cell surface receptors TRAIL-R1 and -R2 are overexpressed by a variety of cancer cell types.

Although TRAIL-based therapies have been currently used mainly in cancer, the therapeutic value of TRAIL-based treatments in autoimmune diseases has been proposed[146]. A number of therapeutic strategies involving TRAIL have been used to treat various experimental autoimmune diseases in animal models *(Figure 8)*[147].

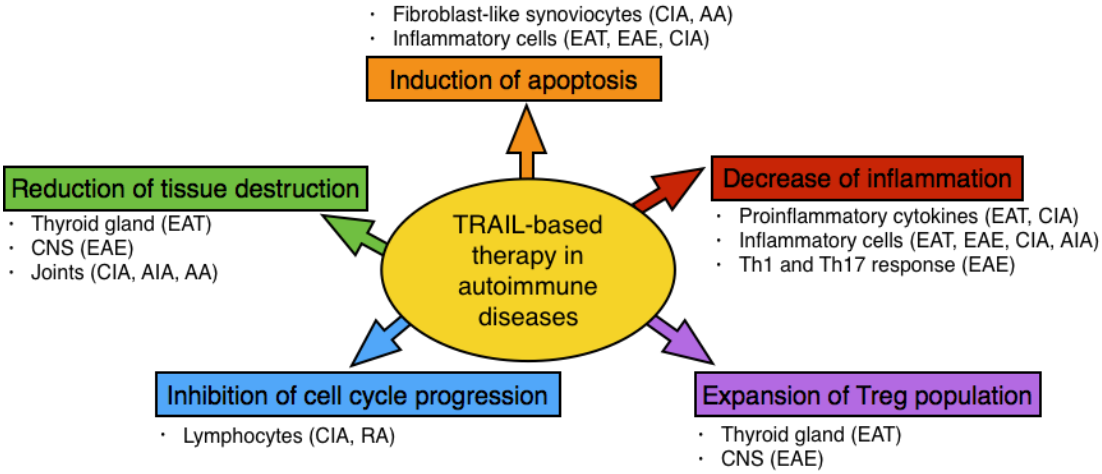


Figure 8 - *Summary of the main effects of TRAIL-based therapy in different experimental models of autoimmune diseases (EAT: experimental autoimmune thyroiditis; EAE: experimental autoimmune encephalomyelitis; CIA: collagen induced arthritis; AIA: antigen induced arthritis; AA: adjuvant arthritis, RA: rheumatoid arthritis)*[147]

In phase 1b and 2 study[148–150], although the combination of Dulanermin with standard chemotherapy did not seem to significantly increase antitumoral activity, it was **well tolerated with no toxicity or adverse effects on patients**[151].

The existence of Dulanermin as a potential therapeutic perspective with a safe profile in diabetes makes more considerable the understanding of TRAIL in the natural history and progression of T1D and its complications.

Dulanermin might be considered as a therapeutic option for the treatment of T1D, either administered alone or in combination with other therapeutic strategies (i.e., immunosuppressant or islet transplantation), in the early onset or in the late phase of T1D, in order to attenuate the autoimmune and the inflammatory response[133].

CHAPTER 2

STUDY I

WHAT HAPPENS TO TRAIL LEVELS IN CHILDREN WITH TYPE 1 DIABETES?

Published in Acta Diabetologica

Tornese, G., Iafusco, D., Monasta, L., Agnoletto, C., Tisato, V., Ventura, A., Zauli, G., Secchiero, P. (2014). The levels of circulating TRAIL at the onset of type 1 diabetes are markedly decreased in patients with ketoacidosis and with the highest insulin requirement. *Acta Diabetologica*, 51(2):239–46.

AIM

The aim of this study was to analyse the serum levels of TRAIL in a paediatric retrospective cohort, mainly including T1D patients and age-matched healthy control subjects.

MATERIALS AND METHODS

Patients and sample collection

Sera of paediatric individuals followed at the Second University of Naples were obtained from patients with T1D (n=387), healthy subjects without islet-specific autoantibodies (n=98, indicated as controls), and healthy subjects with the presence of at least one islet-specific autoantibody (n=22, “AutoAb POS/T1D NEG”).

Among T1D patients, blood was taken at onset (within the first 48 h after diagnosis, n=167) or at later times after onset (n=220). Parents provided informed consent to blood sample drawing and storage for research purposes, in accordance with the Declaration of Helsinki of 1975. Data included age, sex, diagnosis, age at onset (if T1D) or at first visit (if not-T1D), age at blood sample drawing, HbA1c, onset with diabetic ketoacidosis (DKA), islet-specific autoantibodies detected (ICA, GADA, IA2A, IAA, ZnT8A), and the presence of other autoimmune diseases.

After onset, all T1D patients were placed on intensive insulin therapy consisting of three daily regular insulin injections at meals and NPH insulin at lunch and bed time. The regimen was then individually tailored during the follow-up, with the introduction of rapid- and/or long-acting analogues. Insulin daily requirements (in U/Kg/d) were calculated dividing the total daily insulin dose (in units) by patient weight (in kilograms).

Laboratory analyses

A1c values were measured by using a point-of-care platform (DCA-2000 Analyzer, Siemens/Bayer, Munich, Germany). ICA antibodies were measured with an indirect immunofluorescence assay using cryosections of fresh frozen human blood group O pancreas as substrate; GADA, IA2A, and IAA antibodies were detected by radioimmunoassay (RIA) commercial kits (Biochem Immuno-Systems, Italy for GADA and IA-2A; CIS Italy for IAA); ZnT8A antibodies were assessed by ELISA commercial kits (RSR limited, United Kingdom).

Serum TRAIL was measured in duplicate by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Selected samples were run in each ELISA plate as internal controls, confirming the reproducibility of determinations over time.

Statistical analysis

Box plots were used to show the median, interquartiles, and minimum and maximum values for each group of data. After verifying that the TRAIL serum values of this study did not distribute normally (skewness and kurtosis joint normality test), we applied the nonparametric Mann–Whitney test or the Kruskal–Wallis test for the equality of populations to compare the TRAIL values among different populations. The Bonferroni correction was applied if multiple two-by-two comparisons were necessary after conducting a Kruskal–Wallis test. Correlation coefficients were calculated with the Spearman's rank coefficient ρ. A p value <0.05 was considered statistically significant.

In the graphs, horizontal bars are median; upper and lower edges of box are 75^{th} and 25^{th} percentiles; lines extending from box are 10^{th} and 90^{th} percentiles. Asterisk (*) means a p value <0.05.

RESULTS

The cohort of individuals analysed in the present study included 387 patients with T1D and 98 age-matched healthy control subjects. Twenty-two subjects with positivity for autoantibodies, but without overt T1D (AutoAb POS/T1D NEG), were also included *(Table 2)*.

Table 2 - *Characteristics of the subjects included in the study. Continuous variables are presented as median with interquartile range (IQR) in parenthesis; categorical variables are presented either as number or percent unless otherwise indicated. * Subjects positive for autoantibodies (GADA, IA2A, ICA, IAA, or ZnT8A), ** Pathologies of autoimmune origin including Addison's disease, vitiligo, juvenile idiopathic arthritis, multiple sclerosis, psoriasis*

	Controls	AutoAb POS/ /T1D NEG	T1D
Subject number	98	22	387
Sex, female/male (%)	35/65	23/77	44/56
Age at blood drawing (years)	9.2 (5.8-12.0)	11.0 (7.5-13.5)	9.3 (5-4-12.7)
Age at onset (years)	N/A	10.2 (6.4-13.1)	8.6 (4.7-11.8)
A1c (%)	5.0 (4.8-5.2)	5.6 (5.1-5.8)	10.9 (9.3-12.5)
A1c (mmol/mol)	33.3 (31.1-35.5)	36.6 (32.2-39.9)	95.6 (78.1-113-1)
DKA	N/A	N/A	250
*Autoantibodies positivity (%)**	0	100	87
Hashimoto's thyroiditis	0	0	70
Coeliac disease	0	2	22
*Other pathologies of autoimmune origin***	0	0	10

The circulating levels of TRAIL assessed in patients with T1D (median 65.7 pg/ml) were significantly lower ($p<0.001$) when compared with healthy control subjects (median 83.1 pg/ml) *(Figure 9)*.

Moreover, TRAIL levels in T1D patients were significantly lower also when compared to the AutoAbPOS/T1DNEG subjects (median 77 pg/ml; $p=0.030$) *(Figure 9)*. On the other hand, preliminary data indicate that TRAIL levels in paediatric patients affected by maturity-onset diabetes of the young (MODY, n=10) were not significantly different from healthy controls (median 81 pg/ml; $p=0.037$).

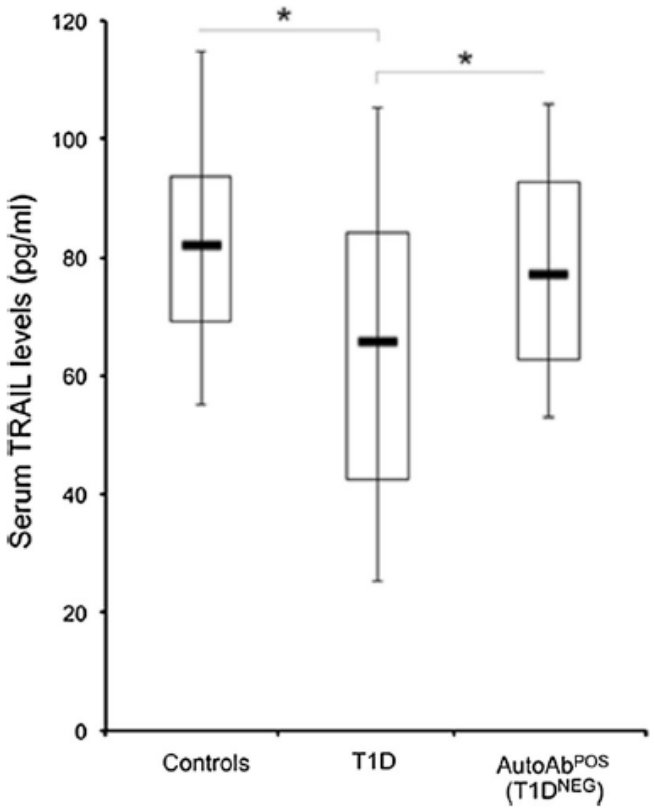


Figure 9 - *Levels of circulating TRAIL in healthy control subjects (controls, n=98), patients with T1D (n=387), subjects with positivity for islet-specific autoantibodies, but without overt T1D (AutoAbPOS/T1DNEG, n = 22).*

The serum levels of TRAIL in the T1D patient cohort were evaluated with respect to relevant demographic, biochemical, and clinical markers. No significant differences were observed in relation to the sex (p=0.409), and no significant correlations were observed between serum TRAIL and age of the patients (ρ=-0.022, p=0.655) as well as between serum TRAIL and HbA1c levels (ρ=-0.068, p=0.353).

Moreover, we excluded the possibility that differences in TRAIL levels could depend from long sample storage, since no significant differences were observed in TRAIL value between samples analysed within 36 months after blood drawing and samples stored for more than 36 months and up to 200 months *(Figure 10)*.

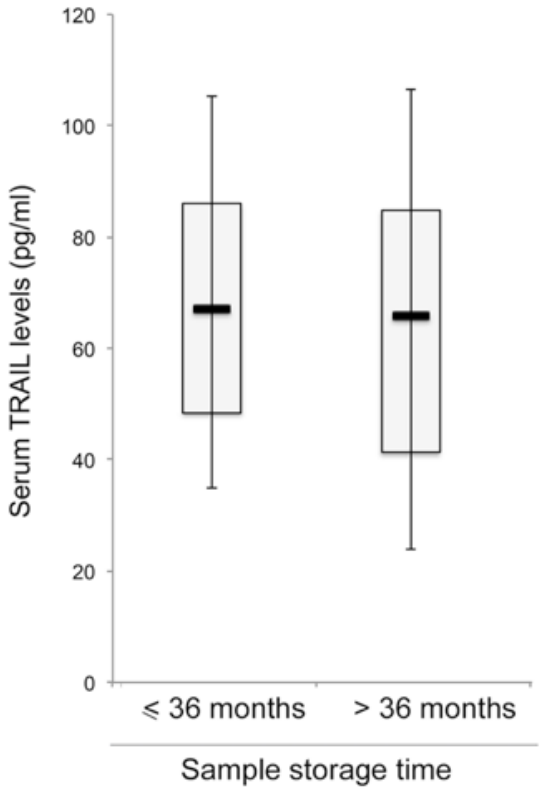


Figure 10 - *Levels of circulating TRAIL according to the sample storage time: within 36 months after blood drawing and samples stored for more than 36 months and up to 200 months.*

Subsequently, in consideration of the role of TRAIL in controlling autoimmunity, we analysed whether TRAIL levels exhibited significant modulation in relationship to the presence of islet-specific autoantibodies (ICA, GADA, IA2A, IAA, ZnT8A). T1D patients with islet-specific autoantibody were 87% and did not differ in terms of circulating TRAIL levels from T1D autoantibody negative *(Figure 11)*. When we analysed the circulating levels of TRAIL in relation to the positivity to each single autoantibody, we found differences close to be significant (p=0.058) only in association with the positivity/negativity for ICA autoantibody, with TRAIL levels lower in the ICA-positive patients (median 61.5 pg/ml) with respect to the ICA-negative ones (median 74.6 pg/ml) *(Figure 11)*.

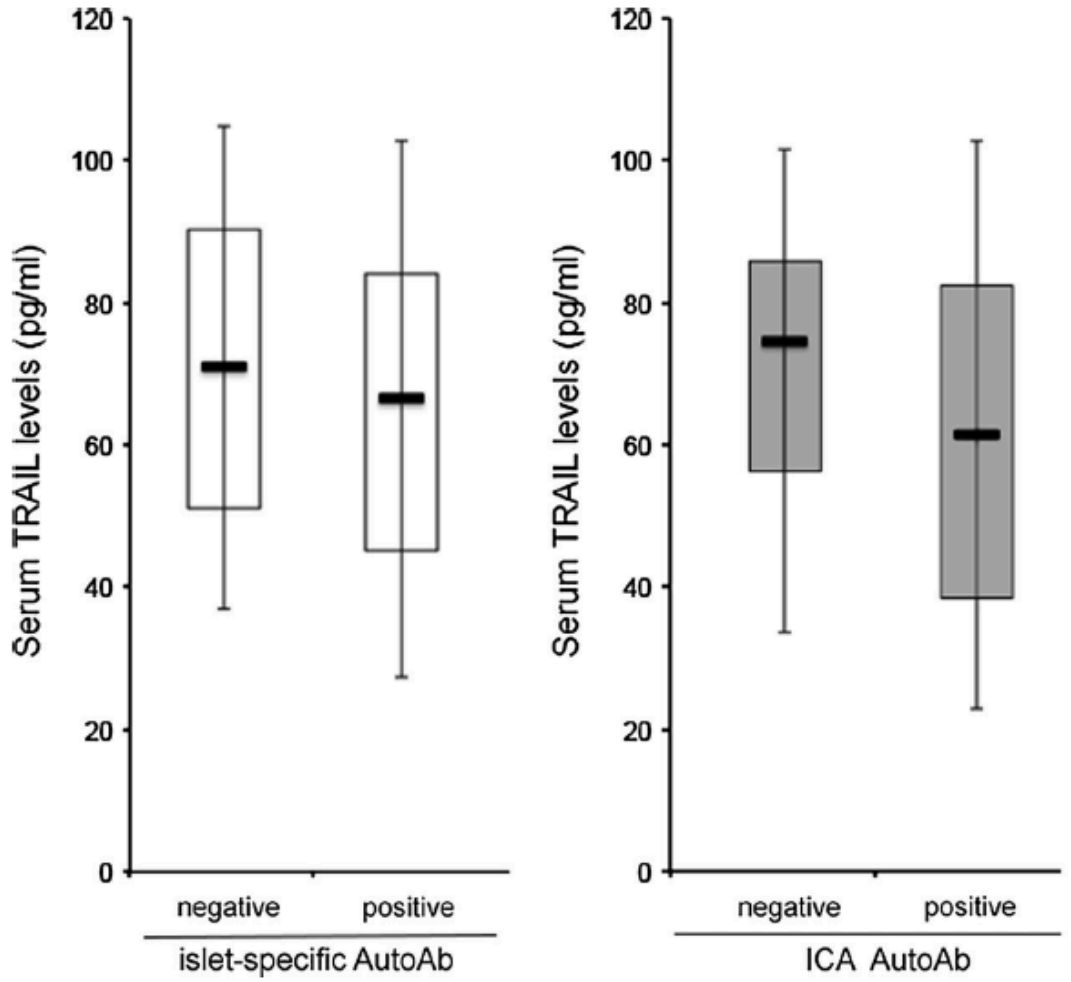


Figure 11 - *Levels of circulating TRAIL in patients with negative (n=41) or positive (n=281) islet-specific autoantibodies (GADA, IA2A, ICA, IAA, or ZnT8A) and in relationship to the positivity or negativity for the ICA autoantibody (AutoAb).*

Moreover, the analysis of TRAIL levels in relation to the presence of other concomitant autoimmune disorders (celiac disease, Hashimoto's thyroiditis, Addison's disease, vitiligo, juvenile idiopathic arthritis, psoriasis, multiple sclerosis) in the T1D patients did not reveal any significant difference, both when considered together *(Figure 12)* or separately (for celiac disease and Hashimoto's thyroiditis).

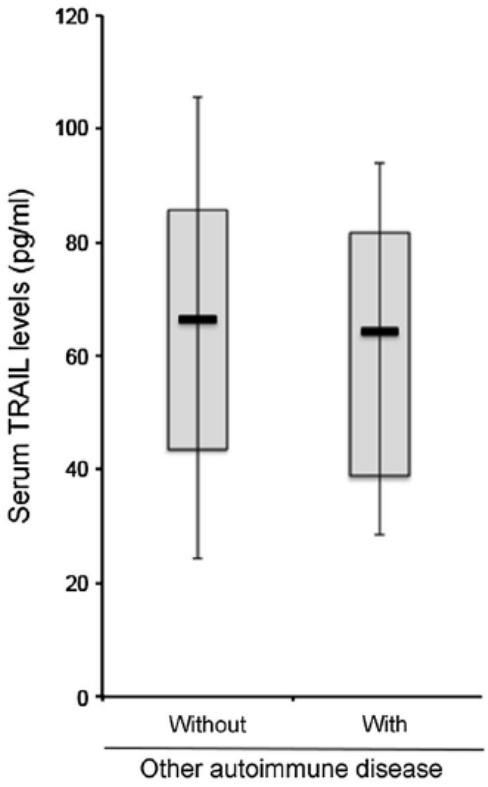


Figure 12 - *Levels of circulating TRAIL in T1D patients with or without other concomitant autoimmune disease(s). The levels of TRAIL were analysed by comparing T1D patients with or without other concomitant autoimmune diseases.*

Since the T1D serum samples assessed derived from blood harvesting performed at different times from T1D onset, we evaluated the levels of circulating TRAIL in relation to the time from diagnosis, by dividing the samples in three main temporal intervals: ‘‘onset’’ (within 48 h from diagnosis; n=167), ‘‘first year’’ (mean 95 days; n=108), and ‘‘> 1 year’’ after diagnosis (mean 483 days; n=112). TRAIL values appear to significantly increase with time of blood drawing from the onset of T1D (Spearman’s rank correlation: ρ=0.183, p <0.001). Kruskal–Wallis equality-of-populations rank test showed a significant difference in TRAIL values among categories (p=0.001). We thus adopted a Mann–Whitney rank-sum test to verify which categories were different from each other, in terms of TRAIL values, applying a Bonferroni correction (p<0.016). TRAIL values at onset (median 61.2 pg/ml) were significantly lower than the levels measured at later time points after diagnosis, both with respect to the ‘‘first year’’ (median 71.3 pg/ ml; p=0.003) and to ‘‘>1 year’’ after diagnosis (median 70.0 pg/m; p=0.002) *(Figure 13)*.

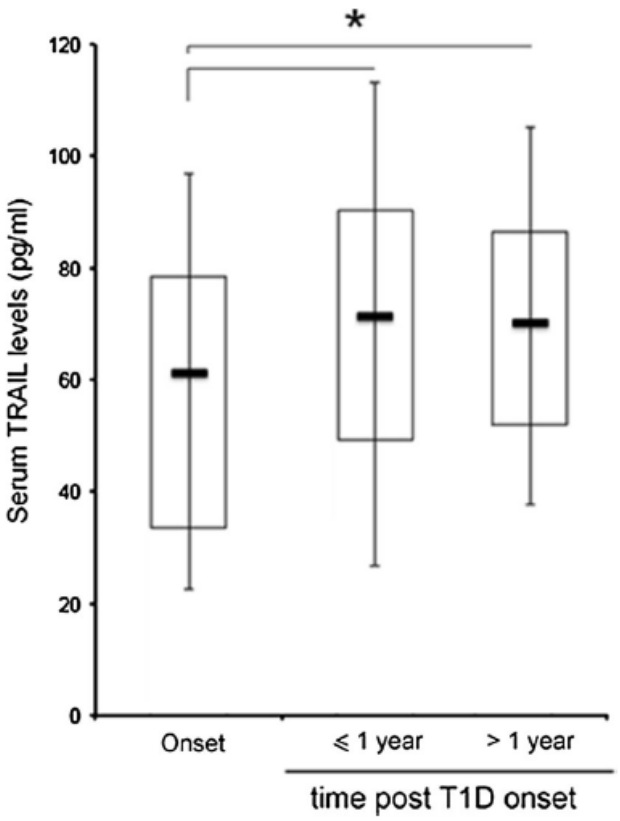


Figure 13 *- The lowest levels of circulating TRAIL are at T1D onset. Serum levels of TRAIL were analysed in relation to the time from diagnosis by dividing the patient samples in three main temporal intervals: "onset" (n = 167), "≤1 year" (n = 108), and ">1 year" (n = 112) after diagnosis.*

Therefore, although reduction in TRAIL levels with respect to healthy controls was marked at onset, it persisted also in patients analysed >1 year from diagnosis.

Subsequently, in order to investigate whether the levels of circulating TRAIL might be related with the severity of the disease, we have analysed the levels of circulating TRAIL at onset in relation to the presence or absence of DKA. In T1D patients with DKA at onset (n= 135), TRAIL levels (median 58.7 pg/ml) were significantly lower ($p<0.0005$) than in patients without DKA at diabetes onset (n=32; median 73.1 pg/ml) *(Figure 14)*.

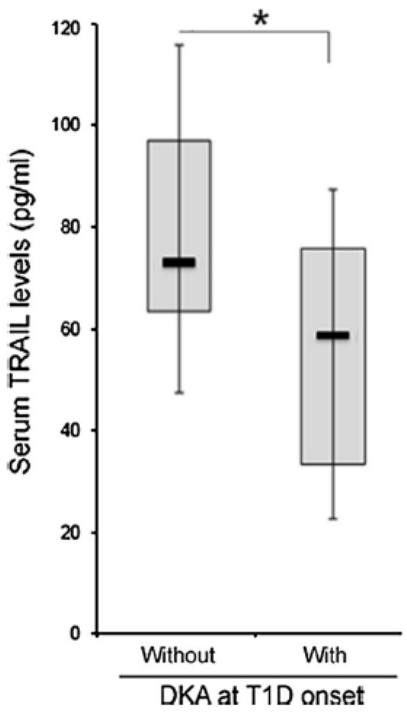


Figure 14 - *The lowest levels of circulating TRAIL are at T1D onset in patients with ketoacidosis. The levels of TRAIL at T1D onset were analysed by comparing patients with (n=135) or without DKA (n=32).*

Moreover, for a subgroup of T1D patients (n=70), for which we had insulin requirements at 3-month intervals up to 21 months of follow-up, we analysed the rank correlation between TRAIL levels at onset and daily insulin requirements. With the exception of the "3 months" time point, the TRAIL levels at onset were significantly and negatively correlated at the insulin daily requirement (U/Kg/day) at all time analysed up to 21 months after discharge *(Table 3)*.

Table 3 - *Insulin daily requirements by 3-month intervals (since onset of T1D and up to 21 months) and correlation with TRAIL levels at onset. Correlation coefficients were determined by Spearman's analysis. (IQR: interquartile range)*

	Insulin daily requirements (U/kg/d) Median (IQR)	***Correlation with TRAIL at onset*** ρ (p value)
Onset	0.71 (0.59-0.90)	-0.36 **(0.001)**
3 months	0.47 (0.39-0.67)	-0.09 (0.451)
6 months	0.46 (0.29-0.58)	-0.29 **(0.015)**
9 months	0.47 (0.29-0.61)	-0.28 **(0.025)**
12 months	0.49 (0.33-0.66)	-0.29 **(0.021)**
15 months	0.50 (0.34-0.72)	-0.27 **(0.025)**
18 months	0.61 (0.46-0.80)	-0.27 **(0.031)**
21 months	0.65 (0.48-0.77)	-0.27 **(0.027)**

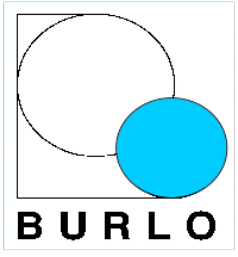
CHAPTER 3

STUDY II

WHAT HAPPENS TO TRAIL LEVELS AT TYPE 1 DIABETES ONSET AND/OR DURING KETOACIDOSIS? AND WHAT THEREAFTER?

Published in Acta Diabetologica

Tornese, G., Tisato, V., Monasta, L., Brumatti Vecchi, L., Zauli, G., Secchiero, P. (2015). Serum TRAIL levels increase shortly after insulin therapy and metabolic stabilization in children with type 1 diabetes mellitus. *Acta Diabetologica,* 52(5):1003-6.



AIM

The aim of this study was to analyse the evolution of circulating TRAIL levels in a pilot group of paediatric patients admitted at Emergency Department for T1D, from the time of hospital admission throughout the re-establishment of a normal metabolic balance and up to 18 months of clinical follow-up.

Moreover, the serum levels of TRAIL in T1D patients were analysed in relation to the metabolic status.

MATERIALS AND METHODS

Patients and sample collection

A total of 80 blood samples were obtained from 11 paediatric patients admitted for T1D onset or secondary diabetic ketoacidosis (DKA) at the Emergency Department of the Institute for Maternal and Child Health ‘‘Burlo Garofolo’’ of Trieste (Italy) *(Table 4)*.

Sample for blood gases was taken at admission to verify the presence of DKA in clinical suspicion of T1D or in patient with pre-existing uncontrolled T1D.

DKA was defined as “mild” when pH was 7.2-7.3 and/or HCO_3 10-15 mEq/l. “moderate” when pH was 7.1-7.2 and/or HCO_3 5-10 mEq/l. and “severe” when pH was <7.1 and/or HCO_3 <5 mEq/l. When DKA was excluded (pH >7.3 and HCO_3 >15 mEq/l). no additional CBG were performed. In case of DKA. patients were treated according to ISPAD guidelines[43]; blood glucose and blood gases were repeated as per protocol until stabilization and serum TRAIL was evaluated in each blood sample.

Table 4 - *Characteristics of the subjects included in the study*

Patient	Sex	Age (years)	Pubertal status	BMI (kg/m^2)	BMI SDS	Admission reason	Type of DKA	Blood glucose (mg/dl)	pH	HCO_3 (mEq/l)	A1c (% - mmol/mol)	C-peptide (ng/ml)	Insulin requirement (units/kg/day)
1	F	14.3	Post-pubertal	26.03	+1.42	Secondary DKA	severe	753	6.91	6	8.0 - 64	N/A	0.65
2	M	13.7	In established puberty	20.20	-0.17	Secondary DKA	mild	291	7.26	14	9.6 - 81	N/A	0.6
3	M	8.6	Pre-puberatal	14.38	-0.62	New onset	moderate	302	7.12	13	13.3 -122	0.26	0.7
4	M	12.2	In established puberty	14.76	-2.06	New onset	moderate	567	7.2	10	11.8 105	0.25	0.85
5	F	7.0	Pre-puberatal	18.94	+0.98	New onset	moderate	343	7.18	11	12.2 - 110	0.35	0.94
6	F	5.8	Pre-puberatal	13.91	-1.38	New onset	mild	570	7.34	11.5	10.7 - 93	N/A	0.97
7	M	12.6	In established puberty	26.05	+1.48	New onset	none	398	7.45	25	8.6 - 70	2.56	0.2
8	F	11.6	In established puberty	16.22	-1.25	New onset	none	593	7.56	24	11.3 - 100	0.67	0.75
9	F	16.8	Post-pubertal	16.10	-2.63	New onset	none	394	7.44	25	8.3 - 67	0.59	0.52
10	M	9.8	Pre-puberatal	14.85	-1.49	New onset	none	270	7.32	17	12.0 - 108	0.21	0.75
11	M	9.8	Pre-puberatal	16.74	-0.45	New onset	none	367	7.41	24	9.7 - 83	0.37	0.31

Once metabolic stabilization has been obtained, all patients were placed or restarted on intensive subcutaneous insulin therapy consisting in “basal bolus” scheme (with 3 daily rapid-acting analogue insulin injections at meals and 1 long-acting analogue insulin at bedtime) to reach optimal glycaemic levels

Insulin daily requirement (in U/kg/day) was calculated dividing the total daily insulin dose (in Units) at discharge by patient weight (in kilograms).

A blood sample was taken to determine TRAIL levels before hospital discharge and additional blood samples were taken from each patient before hospital discharge and every 6 months during the clinical follow-up.

For all patients (including patient 7 which was overweight [>85th centile] and had high non-fasting C-peptide levels) the presence of diabetes-associated autoantibodies together with the lack of signs of insulin resistance (i.e. acanthosis nigricans), reasonably excluded T2DM. as well as monogenic diabetes

Parents provided informed consent to blood sample drawing for research purposes. in accordance with the Declaration of Helsinki of 1975. The study was approved by the Bioethics Committee of the IRCCS ‘‘Burlo Garofolo’’ (Trieste, Italy; RC 18/13).

Laboratory analyses

Blood gases were measured by using ABL800 Flex (Radiometer, Brønshøj. Denmark).

Blood glucose level was measured via a hexokinase enzymatic reaction by Cobas 6000 (Roche Diagnostics, Indianapolis, IN, USA).

A1c was assessed using turbidimetric inhibition immunoassay by Cobas Integra 400 Tina-quant Hemoglobin A1c Gen.2 (Roche Diagnostics,

Indianapolis, IN, USA).

C-peptide was estimated using a validated chemiluminescent assay on the Immulite 2000 (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Serum TRAIL was measured in duplicate by using a commercially available ELISA kit (R&D Systems. Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical analysis

Differences between values at two different time points were evaluated with a pairwise sign-rank Wilcoxon's test. Correlation coefficients were calculated with the Spearman's rank coefficient ρ. A p value <0.05 was considered statistically significant, after applying a Bonferroni correction if multiple rank-correlations were calculated simultaneously.

In the graphs, horizontal bars are median; upper and lower edges of box are 75^{th} and 25^{th} percentiles; lines extending from box are 10^{th} and 90^{th} percentiles. Asterisk (*) a p value <0.05.

RESULTS

The main demographical and clinical characteristics of the paediatric patients enrolled in the present study are summarized in *Table 4*.

Comparative analysis of the circulating TRAIL levels, showed median TRAIL level at admission of 52.8 pg/ml, with the lowest levels measured in the patients with DKA (median 47.9 pg/ml) with respect to patients without DKA (median 76.14 pg/ml).

Of interest, in all patients included in the study, we documented a significant increase of TRAIL levels ($p<0.01$, using the sign-rank pairwise Wilcoxon test) at the time of discharge (median of time between the two measurement was 2,5 days) with a median value of 100.3 pg/ml *(Figure 15)*. A further significant ($p<0.05$) increase was documented after 6 months, reaching median values of TRAIL of 147.3 pg/ml that were maintained without significant modulations also in the subsequent time points/assessments up to 18 months *(Figure 16)*.

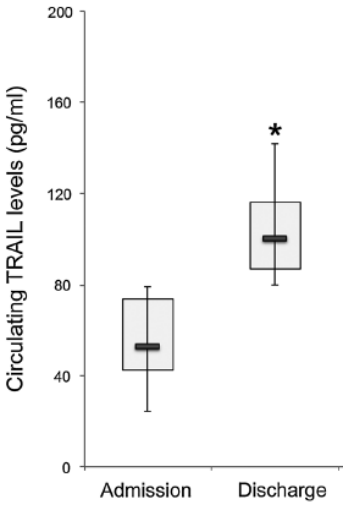


Figure 15 - *Circulating TRAIL levels were monitored in serum samples collected at the admission and discharge in patients admitted to Emergency Department with T1D at onset and/or DKA.*

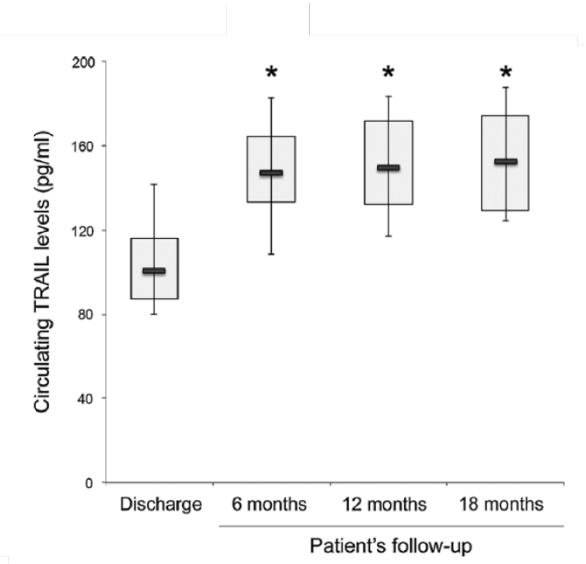


Figure 16 - *Circulating TRAIL levels in serum samples collected at the indicated time points of the clinical history of the T1D paediatric patients enrolled in the study.*

As for clinical protocol, in the patients with DKA (n=6) upon admission between 5 and 10, blood samples were taken for each subject before reaching normalization of venous blood gas and/or blood glucose. This allowed us to monitor TRAIL levels also at the time points corresponding to metabolic stabilization (median time between admission and stabilization of 15.5 h; IQR 10.1–20.9). Analysis of TRAIL levels in relationship with all the clinical/metabolic parameters revealed a significant ($p<0.05$) correlation only between TRAIL levels and HCO_3 (Spearman's rank correlation, $\rho=0.4944$, $p=0.0014$), BE ($\rho=0.4405$, $p=0.0044$), pH ($\rho=0.368$, $p=0.0193$), pCO_2 ($\rho=0.3962$, $p= 0.0114$) *(Figure 17)*.

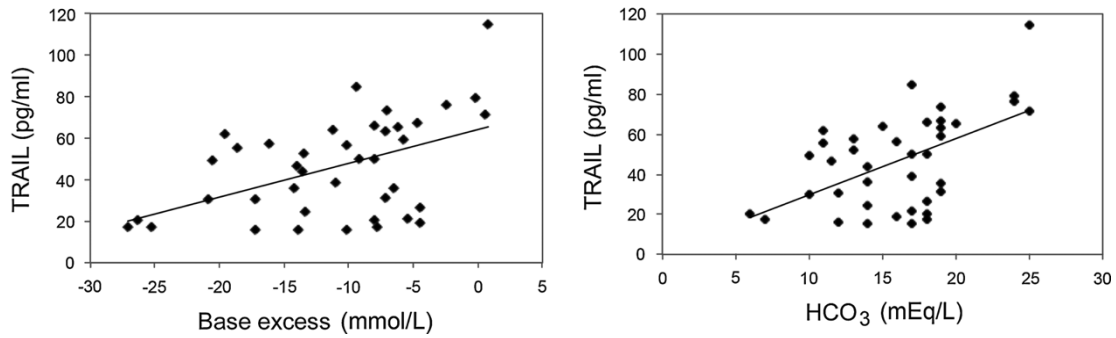


Figure 17 - *Correlation analyses between TRAIL circulating levels and clinical parameters related to the metabolic status of patients. Positive correlation between TRAIL and base excess (BE) as well as between TRAIL and bicarbonate (HCO_3) are shown*

CHAPTER 4

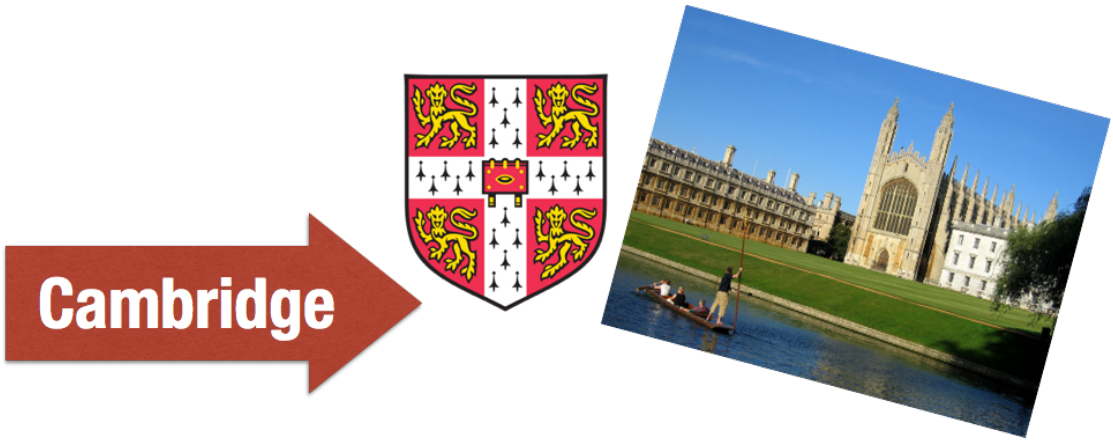
STUDY III

WHAT HAPPENS IN LONG-STANDING TYPE 1 DIABETES? ARE TRAIL LEVELS CORRELATED WITH MARKERS OF RESIDUAL B-CELL MASS, INFLAMMATION OR AUTOIMMUNITY?

Manuscript in preparation

Tornese, G., Marcovecchio, M.L., Wicker, L.S., Secchiero, P., Todd, J.A., Dunger, D.B. Does TRAIL relate to measures of beta-cell function, general inflammation and immune activation in young people with T1D?

This study was supported by the JDRF, the Wellcome Trust (061858, 076113 and 091157), the National Institute for Health Research Cambridge Biomedical Research Centre and the JDRF UK Centre for Diabetes – Genes, Autoimmunity and Prevention Grant (4-2007-1003). The research leading to these results has received funding from the European Union's 7th Framework Programme (FP7/2007-2013) under grant agreement no. 241447 (NAIMIT). The Cambridge Institute for Medical Research (CIMR) is in receipt of a Wellcome Trust Strategic Award (100140). The NFS is funded by the JDRF, the Wellcome Trust and Diabetes UK.



AIM

The aims of this study were:

(1) to determine factors modulating levels of circulating TRAIL and over time changes in TRAIL;

(2) to assess whether there was an association between TRAIL and residual beta-cell function, as assessed by C-peptide levels;

(3) to assess whether TRAIL relate to other inflammatory/immune markers, in particular high-sensitivity CRP (hs-CRP) and soluble CD25 (sCD25).

MATERIALS AND METHODS

Patients and sample collection

Four hundred fifty-one non-fasting serum samples for measuring TRAIL, sCD25, hsCRP and C-peptide levels were available from 232 young people followed in the Nephropathy Family Study (NFS). The NFS is a prospective study that has, since 2000, recruited more than 1,000 adolescents (10–18 years) with T1D and has followed them longitudinally [152]. For the present analysis, 219 patients had two available stored serum samples collected at a median time interval of 1.2 years [1.0-1.7]. No significant differences were found in age at diagnosis, sex distribution and glycemic control between selected subjects and the entire cohort.

Ethics approval was obtained from the ethics committee, with written consent from participants or parents with assent from the children. All data and samples are treated as confidential. Samples and data are identifiable by a unique barcode only, and volunteers are free to withdraw from these projects at any time.

Laboratory analyses

All plasma and serum samples were stored at –80°C until analyses were performed. sCD25 and C-peptide were already determined for a previous study[153].

Serum TRAIL was measured on frozen serum aliquots by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions, as previously described[154]. The lower limit of detection of this assay was 15.6 pg/ml.

hs-CRP (Cardiac C-Reactive Protein) was run on the Siemens Dimension RXL auto analyser. The lower limit of detection of this assay was 0.1 mg/l and hs-CRP levels >10 mg/l were excluded from the analysis, as they are likely related to infections or other acute inflammatory processes [155,156].

Since circannual rhythms have been described to significantly increase CRP concentration[157] and a seasonal variation of the incidence of T1D in children under 15 years of age is a known phenomenon [158] we also considered seasonal variation of variables (as a 4-level factor variable, using the 21st day of June, September, December and March as cut-off to define summer [1], autumn [2], winter [3] and spring [4]).

Statistical analysis

Data are mean ±SD or median [interquartile range].

Differences between values at two different time points were evaluated with a pairwise sign-rank Wilcoxon's test.

Due to non-normal distribution, TRAIL, A1c, C-peptide, sCD25, and hs-CRP were Log (10) transformed for analyses requiring normal distribution. Association between variables was performed with repeated measures logistic regression using Generalized estimating equations (GEE) to account for the lack of independence when two samples were available for the same subject and results are expressed as β coefficients.

Changes over time (Δ) of variables were calculated as: (variable at time 2 – variable at time 1)/variable at time 1. Association between Δ of variables was performed with linear regression analysis and results are expressed as β coefficients.

SPSS version 20 was used for the analyses. Statistical significance was defined by a p values <0.05.

RESULTS

Two hundred thirty-two subjects were enrolled in the present study (59% female), with a median age at diagnosis of 8.9 years [5.3-11.1]. Two hundred nineteen subjects had two available samples and the median time between the two visits was 1.2 years [1.0-1.7] *(Table 5)*.

Table 5 - *Demographical and clinical characteristics of the cohort at the 2 visits. Data are mean ±SD or median [interquartile range]*

	Visit 1	Visit 2	p
Age (yr)	14.5 ±2.3 [range: 10.1-20.1]	16.0 ±2.3 [range: 11.4-21.8]	**<0.001**
Duration of disease (yr)	5.5 [3.4-9.0]	7.1 [4.9-10.2]	**<0.001**
BMI SDS	0.8 ±1.0	0.8 ±1.0	0.278
A1c (%)	8.7 [8.0-9.8]	9.0 [7.9-10.1]	0.398
TRAIL (pg/ml)	78.6 [65.9-97.6]	73.0 [56.8-92.1]	**0.001**
sCD25 (pg/ml)	3183 [2546-3903]	2873 [2263-3634]	**<0.001**
hs-CRP (mg/dl)	0.6 [0.2-1.3]	0.6 [0.2-1.8]	**0.040**
C-peptide (pmol/l)	23.8 [9.5-65.1]	22.1 [10.5-59.1]	**<0.001**

Log TRAIL levels were inversely correlated with age at bleed ($p=1.05 \times 10^{-7}$), duration of disease ($p=6.56 \times 10^{-6}$) and seasonality ($p=1.42 \times 10^{-4}$) (with spring as the most associated and summer the least) *(Table 6)*. A significant (p=0.001) decrease between visit 1 and visit 2 was detected for TRAIL *(Table 5)*.

Table 6. *Clinical and biochemical variables associated with Log TRAIL. Results are adjusted for repeated measure using GEE model.*

	B (95% CI)	***p value***
Age at bleed	-0.021 (-0.028, -0.013)	**1.05×10^{-7}**
Duration of disease	-0.012 (-0.018, -0.007)	**6.56×10^{-6}**
Seasonality	0.036 (0.017, 0.054)	**1.42×10^{-4}**
Age at diagnosis	0.002 (-0.003, 0.008)	0.463
Log A1c	0.055 (-0.221, 0.331)	0.697
BMI SDS	0.001 (-0.18, 0.021)	0.907
Female Sex	0.036 (-0.004, 0.077)	0.076

The reduction over time in C-peptide (used as marker of beta-cell function) was significant from visit 1 (median 23.8 pmol/l [9.5-65.1]) to visit 2 (median 22.1 pmol/l [10.5-59.1]) ($p<0.001$) *(Table 5)*. However, no association was found between Log TRAIL and Log C-peptide (β coefficient=0.015, p=0.472) *(Table 7)* and the association between Δ TRAIL and Δ C-peptide did not reach statistical significance (β coefficient=0.024, p=0.068).

Table 7. *Independent associations between TRAIL and markers of beta cell function and immune and inflammatory markers. Each model was built separately and results are adjusted for seasonality, sex, BMI SDS, Log A1c, age at diagnosis and duration of T1D and repeated measure using GEE model.*

	B (95% CI)	***p value***
Model 1: β-cell function marker		
Log C-peptide	-0.015 (-0.054, 0.025)	0.472
Model 2: inflammation marker		
Log hs-CRP	0.014 (-0.033, 0.060)	0.557
Model 3: immune system marker		
Log sCD25	0.231 (0.094, 0.368)	**0.001**

The inflammatory marker, hs-CRP, increased significantly (p=0.040) over time, from visit 1 (0.6 mg/dl [0.2-1.3]) to visit 2 (0.6 mg/dl [0.2-1.8]) *(Table 5)*. However Log TRAIL was not associated with Log hs-CRP (β coefficient=-0.014, p=0.557) *(Table 7)* and the association between Δ TRAIL and Δ hs-CRP was not significant (β coefficient=0.068, p=0.857).

The immune marker, sCD25, increased significantly (p<0.001) over time, from visit 1 (median 3183 pg/ml [2546-3903]) to visit 2 (median 2873 pg/ml [2263-3634) (p<0.001) *(Table 5)*.

Log TRAIL was associated with Log sCD25 (β coefficient=0.222, $p=3.55 \times 10^{-4}$) *(Table 7)*, even after adjustment for seasonality, sex, BMI SDS, Log A1c, age at diagnosis and duration of T1D (β coefficient=0.231, p=0.001); age at bleed was not included among adjustments because of collinearity with duration of disease (Spearman's rho=0.3748, p<0.001).

Also Δ TRAIL was associated with Δ sCD25 (β coefficient=0.348, $p=3.02 \times 10^{-5}$), even when adjusted for time between visits (β coefficient=0.349, $p=3.08 \times 10^{-5}$).

CHAPTER 5

GENERAL DISCUSSION

1. TRAIL IN TYPE 1 DIABETES

In study I we measured TRAIL in a paediatric retrospective cohort, mainly including T1D patients and age-matched healthy control subjects[154]. Patients with T1D had a mean age of 9.3 years, with a mean age at diagnosis of 8.6 years, including both recent onset and overt T1D (mean glycosylated haemoglobin [A1c] 10.9% – 95.6 mmol/mol).

We have demonstrated that **circulating levels of TRAIL were significantly decreased in patients with T1D** (median 65.7 pg/ml) **with respect to healthy control subjects** (median 83.1 pg/ml) *(Figure 9)*.

These data are consistent with animal studies – which showed that systemic TRAIL blockade as well as knocking out TRAIL gene would increase both incidence and severity of autoimmune diabetes in genetically prone mice[87,130,134] – and with a previous study on diabetic patients (50 patients with age up to 52 years, mean duration of disease 9 years, mean A1c 7%) in which TRAIL was significantly decreased in comparison with age- and sex-matched healthy controls[144]. In this study, the decoy/death receptor ratio was significantly increased in T cells of diabetic patients, probably leading to hyper-activation of T cells and the consequent uprising of auto-reactive T cells which would act against susceptible self tissues. The increased expression of TRAIL-R3 and TRAIL-R4 on T cells from diabetic patients might hinder these cells from inhibitory effects of TRAIL[144].

Lower levels of serum TRAIL in diabetic patients could be causative in pathogenesis of autoimmunity[144]: as cleavage of surface TRAIL from T-cells is considered to be one of the main sources of serum TRAIL[159], this decrease may be the result of more stabilized surface TRAIL. Then **a decrease in circulating TRAIL might lead to autoreactive T-cells uprising**.

On the contrary, many studies have shown that **soluble TRAIL causes cell cycle arrest and blocks cytokine production of autoreactive T cell**s[121] and in this regard it is a prototype of autoimmune inflammation inhibitor[117].

We then confirmed reduced TRAIL levels in children with T1D compared to unaffected individuals.

Moreover, in study III we reported the existence of a significant seasonal pattern of TRAIL, with the lower levels during summer and higher during spring, which may have some impact on the seasonal variation in the initial presentation of T1D[158] and should be taken into account when planning further studies on TRAIL.

2. TRAIL AND AUTOIMMUNITY

Because of the well-documented immunoregulatory role of TRAIL in autoimmune diseases, in study I we investigated some indirect signs of autoimmunity in patients with T1D[154].

Firstly, circulating levels of **TRAIL in healthy autoantibody-positive individuals** (AutoAb POS/T1D NEG) (median 77 pg/ml), who have a higher risk of developing T1D than autoantibody-negative individuals do[35,36], **were not different with respect to healthy control subjects** (median 83.1 pg/ml) *(Figure 9)*.

Secondly, **T1D patients with islet-specific autoantibody** (87% of total) **did not differ from T1D autoantibody negative** in terms of circulating TRAIL levels *(Figure 11)*.

Thirdly, **TRAIL levels in relation to the presence of other concomitant autoimmune disorders** (in 26.5% of the patients, mostly Hashimoto's thyroiditis [68%], coeliac disease [21%]) in the T1D patients **did not reveal any significant difference**, both when considered together *(Figure 12)* or separately (for celiac disease and Hashimoto's thyroiditis).

These observations were against the hypothesis that a decrease in circulating TRAIL has some relationship with the severity of autoimmune reaction characterizing T1D.

Two recent studies on other autoimmune diseases confirmed the incongruity of data regarding autoimmunity:

- in patients with newly diagnosed **Hashimoto's thyroiditis** (positive for both anti-thyroid peroxidase and anti-thyroglobulin antibodies) with subclinical hypothyroidism, circulating TRAIL levels were significantly lower than in controls (67.2 vs. 78.5 pg/ml)[115]

- in patients with **coeliac disease** (CD) at onset no significant differences were found in the circulating levels of TRAIL with regard to patients with either eosinophilic esophagitis or potential CD. Gluten-free diet did not significantly modify the levels of circulating TRAIL at 6 or 12 months. However, patients with CD associated with other auto-immune diseases (10%) showed significantly lower levels of TRAIL when compared with patients with CD alone (86 vs. 100 pg/ml).

Because of this inconsistency, in study III we purposely aimed to assess the potential association between TRAIL and a measure of immune activation. In particular, we chose **soluble CD25 (sCD25)** (also known as **IL-2 receptor α [IL-2RA]**) which is an established marker of immune activation and inflammation, because of its known link with TRAIL[134,160].

IL-2RA subunit is essential for high-affinity binding of IL-2, and unlike the IL-2RB subunit and the common cytokine receptor γ chain, which bind to other cytokines, the α subunit is unique to IL-2[161]. The IL-2/IL-2RA signalling pathway is essential for the regulation of immune responses. Rare IL-2RA mutations cause systemic autoimmune disease in humans[162,163].

Elevated sCD25 concentrations in adults are associated with activation of lymphocytes during autoimmune disease[164–166], thus sCD25 has been used as a biomarker to help characterize disease progression, prognosis and treatment[166–168]. In a recent paper sCD25 was found to be increased in T1D patients and inversely associated with C-peptide, probably reflecting the adverse effects of an ongoing, actively autoimmune and inflammatory immune system on β-cell function in patients[153].

We then analysed TRAIL and sCD25 in a retrospective cohort study on 232 young patients (mean age 14.5 years) with a long-standing T1D (median duration of disease 5.5 years) and a poor controlled diabetes (mean A1c 8.7% – 72 mmol/mol) and a **significant association** was found **between TRAIL and sCD25**.

The majority of these patients (n=219) had 2 available stored non-fasting serum samples collected (median of time interval between the 2 visits: 1.2 years), and a significant reduction in sCD25 was found over time, possibly reflecting a reduced immune activation in T1D, which is generally supported by the finding of reduced levels of autoantibodies decrease with duration of diabetes[169,170].

The association between TRAIL and CD25 was confirmed both as single values and as changes over time.

IL-2 appears to be the dominant cytokine in reprogramming "helpless" $CD8^+$ T cells to β-cell specific cytotoxic lymphocytes during antigenic restimulation, significantly (but not completely) decreasing the production of TRAIL[160]. The recovery of functional $CD8^+$ T cell immunity by IL-2 is concomitant with induction of CD25 expression and down-regulation of TRAIL[160]. On the other hand, TRAIL inhibits in the NOD mouse the proliferation of diabetogenic T-cells by suppressing IL-2 production and cell cycle progression, and this inhibition can be rescued in the presence of exogenous IL-2[134].

The link found between TRAIL and CD25 across age and duration of T1D could therefore imply that the **observed down-regulation of TRAIL reflects the active autoimmune inflammatory process mediated by IL-2**, even years after the onset of diabetes.

3. TRAIL AT THE ONSET OF TYPE 1 DIBAETES

In study I, evaluating the levels of circulating TRAIL in relation to the time from diagnosis, **TRAIL values at "onset"** (median 61.2 pg/ml) **were significantly lower than the levels measured at later time points after diagnosis**, both with respect to "≤1 year" (median 71.3 pg) and to ">1 year" after diagnosis (median 70.0 pg/ml)[154] *(Figure 13)*.

Among T1D patients with blood taken at onset, **TRAIL levels were significantly lower in patients with DKA** (median 58.7 pg/ml) than in patients without DKA at diabetes onset (median 73.1 pg/ml) *(Figure 14)*.

Unfortunately, the first study just evaluated a retrospective cohort with no information about concurrent metabolic status at time of blood sampling.

Hence study II was prospective on a cohort of pediatric subjects admitted for T1D onset or secondary DKA at the Emergency Department[171]. Similarly, in this cohort the **lowest levels of TRAIL were measured in patients with DKA** (median 47.9 pg/ml) with respect to patients without DKA (median 76.14 pg/ml), confirming the previous results.

Analysis of TRAIL levels in relationship with all the clinical/metabolic parameters revealed a **significant correlation** only **between TRAIL levels and HCO_3, BE, pH and pCO_2** *(Table 3; Figure 17)*. **No correlation** was found between TRAIL levels and **C-peptide, A1c, blood glucose** or **insulin daily requirement at discharge from the hospital**.

In all patients, we documented a **significant and prompt increase of circulating TRAIL levels from admission** (median 52.8 pg/ml) **to the time of discharge** (median 109.8 pg/ml) over a median time of 2.5 days *(Figure 15)*.

Since the lowest levels of TRAIL were documented in T1D patients with DKA at the onset, these data suggest a link between circulating TRAIL and the severity of the disease. The fact that TRAIL levels are lower in patients with DKA (at onset or secondary) and are correlated with metabolic parameters,

points to a **possible association of TRAIL with the metabolic stress underlying T1D**.

These data are in agreement with recent studies performed on T2D, which showed that circulating levels of TRAIL were significantly decreased in newly diagnosed T2D patients when compared to controls, and increased after 6 months of diabetic treatment[172].

The fact that both types of diabetes at diagnosis are characterized by low levels of circulating TRAIL suggests that also **metabolic endangerment** – other than autoimmunity – could be a preponderant cause for the decrease in the circulating levels of TRAIL. However, metabolic status cannot be the only cause for reduction in TRAIL levels: TRAIL persists lower than controls levels even a long time after treatment with insulin or metformin has been started and good metabolic status has been steadily reached. Moreover TRAIL is not related to A1c levels.

Patients with DKA are supposed to have lower residual β-cell mass (1-30%)[173], while in many patients levels of stimulated C-peptide at diagnosis are only partially reduced[174,175]. The improvement in C-peptide responses that have been identified in the first months after diagnosis in some individuals suggests that repair of the loss of β-cell function and/or mass may occur early in the course of the disease, either by halting the progression of autoimmune process or by renewing β-cells, and that TRAIL might be related to these changes[176]. However **TRAIL seems not to be related to C-peptide, as an estimation of residual functional β-cell mass**, although in study II subjects might have been too few to find a significant association and in study III data related to long-standing T1D patients might have been biased by absent C-peptide secretion.

A potential molecular mechanism that links metabolic state and circulating levels of TRAIL could be represented by CRP. In individuals with pre-diabetes (both type 1 and type 2) **inflammatory processes** might influence β-cell function and glycaemic control[177]. Indeed, it has been shown that CRP is elevated in children with newly diagnosed T1D and DKA crisis and that it likely

enhances inflammation process by modulating a variety of cytokines[178]. In children with moderate to severe DKA at T1D onset, the presence of increased levels of CRP (without the presence of infection) were reversed with insulin administration in 24 hours, indicating the anti-inflammatory role of insulin[178]. Likewise, it has been documented that TRAIL expression and release is down-regulated by CRP. However **no association was found between TRAIL and CRP in T1D** in study III. Given that those data were related to patients with established T1D with variable duration of the disease instead of recently diagnosed ones, all these effects might have been lost after many years of disease, whereas they might be relevant in the early stage of disease.

One more explanation could be an **early and persistent microvascular damage** in diabetes mellitus. As a matter of fact, in the study on T2D patients, TRAIL levels were positively associated with endothelial function evaluated with flow-mediated endothelium-dependent arterial dilatation (FMD)[172]. This finding has been replicated in a cohort of patients with subclinical hypothyroidism: circulating TRAIL levels were positively associated with endothelial function[179], thus suggesting that **TRAIL levels may be a protective marker of endothelial function**[180].

Consistent with this findings, in patients with proliferative diabetic retinopathy soluble TRAIL levels measured in the conjunctival sac were significantly lower with respect to both patients without retinopathy and patients with non-proliferative diabetic retinopathy. A decreased production and/or release of TRAIL might then contribute to worsening proliferative diabetic retinopathy, possibly by reducing the degree of apoptosis in retinal endothelial cells[181]. Moreover, in mice TRAIL deficiency contributes to diabetic nephropathy with an increased extracellular matrix, mesangial expansion and mesangial cell proliferation in the glomeruli and disorganisation of tubular epithelium, with increased fibrosis[182].

Future longitudinal studies are needed to understand TRAIL modulation in T1D, particularly in relation with endothelial function (at onset and during follow-up)

and with microvascular complications (such as retinopathy, nephropathy and neuropathy).

4. TRAIL IN LONG-LASTING TYPE 1 DIABETES

As already reported, in study I **TRAIL levels increased with time**. TRAIL values from 48 hours until 1 year after diagnosis (“≤1 year”) and thereafter (“>1 year”) were significantly higher than values at onset (median 71.3 pg and 70.0 pg/ml, respectively, vs 61.2 pg/ml). Nevertheless, **also in patients analysed more than 1 year after onset TRAIL levels were still significantly lower with respect to healthy controls** (median 83.1 pg/ml)[154].

Because of the retrospective nature of the study, there was no chance to sequentially evaluate modulation TRAIL levels in the same patients over time.

In study II, by contrast, we could follow prospectively the same patients from the onset of T1D onwards. After a significant increase of circulating TRAIL levels during the first days of hospitalization, we documented a **further significant increase from hospital discharge** (median 109.8 pg/ml) **to the 6-month follow-up** (median 147.3 pg/ml). TRAIL levels were then maintained **without significant modulations in the subsequent time points/assessments up to 18 months**[171] *(Figure 16)*.

These data confirmed that the improvement in TRAIL could be seen soon after commencement of treatment (as already demonstrated in T2D)[172].

Nevertheless study III showed that **in patients with long-standing T1D** (median duration of disease 5.5 years), **TRAIL levels significantly decreased** from the first visit (median 78.6 pg/ml) to the second one (median 73.0 pg/ml), with a median time of 1.2 years between the two visits. The observed reduction in TRAIL over time was **independently associated with lowering of sCD25** (independently from the age, duration of disease and seasonality). As already reported, this reduction in sCD25 might reflect a reduced immune activation occurring in T1D with duration of disease[169,170]. By contrast, no association was found between TRAIL and C-peptide or hsCRP in long-lasting T1D *(Table 7)*.

For a subgroup of T1D patients in study I, for which we had insulin requirements at 3-month intervals up to 21 months of follow-up, we analysed the rank correlation between TRAIL levels at onset and daily insulin requirements up to 21 months after onset. With the exception of the ‘‘3 months’’ time point, **TRAIL levels at onset were significantly and negatively correlated at the insulin daily requirement** (U/Kg/day) at all time analysed **up to 21 months after discharge**.

The **significant surge in TRAIL levels** shortly after short-standing insulin treatment has been established in newly diagnosed T1D or in secondary DKA and the further significant increase in the following 6 months **call to mind the increasing concentrations of circulating C-peptide** and the lower insulin requirement that take place soon after clinical diagnosis (**“honeymoon” period**)[52–56]. Although no associations were found between TRAIL and C-peptide in T1D at onset (in study II) or in long-standing disease (in study III), this could be due to scarcity of subject and exhausted β-cells, respectively. On the contrary the negative correlation between TRAIL and insulin requirement up to 21 months after onset would be in favour of an association between TRAIL and β-cells residual function. In this regard, TRAIL at onset may predict insulin requirement in the medium term and could be used as a predictive biomarker.

Future longitudinal studies are needed to further investigate TRAIL levels in relation to β-cell function: the chance to use recombinant TRAIL to induce immune tolerance against residual β-cell, halt autoimmune destruction and preserve C-peptide make this line of research particularly interesting.

CONCLUSION AND PERSPECTIVES

We showed for the first time the behaviour of circulating TRAIL in paediatric patients with T1D, both at onset and years after onset, in 3 different cohorts.

While observations in patients with long-standing T1D strengthen the link between TRAIL and immune activation, in recent-onset patients TRAIL is strongly linked with metabolic status. Although preliminary experimental data suggested a potential beneficial effect of TRAIL on β-cell survival, we could not find any association between TRAIL and C-peptide.

Future studies are needed to investigate the association of TRAIL with:

- immune activation and general inflammation in children with recent onset of T1D;

- residual β-cell mass at onset and throughout remission period;

- immune activation, general inflammation and residual β-cell mass in children with T2D;

- endothelial function and long-term complications in long-standing T1D.

Whether Dulanermin (recombinant TRAIL) – which has shown a safety profile in several clinical studies – will have therapeutic perspective remains an open question, which requires additional preclinical studies.

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